Genetics, statins, and lipid metabolism in cardiovascular disease

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CHAPTER 1

General introduction and outline of this thesis

General introduction

Cardiovascular disease (CVD) is one of the leading causes of death in industrialized countries. In 2012, 28% of deaths in women and 27% of deaths in men were due to CVD [1]. In addition, it is estimated that the prevalence of CVD in the United States in 2008 was 82.6 million individuals (36.2% of the total population above 20 years) [2]. Although the absolute number of deaths due to CVD has decreased during the last decades, the public health burden from CVD remains high [1;2]. With increasing age, the proportion of deaths caused by CVD increases; in women and men between 55 and 85 years 25-26 % of the deaths can be contributed to CVD, compared to 32-35% in men and women aged 85 years and older [1].

A high low-density lipoprotein (LDL)-cholesterol level is one of the most important risk factors for CVD. Accumulation of lipids and fibrous elements in the large arteries results in atherosclerosis, a progressive disease and an important contributor to CVD [3]. Analysis of 68 prospective studies including over 300,000 participants has shown a strong association between high LDL-cholesterol levels and increased risk for coronary heart disease [4]. Therefore, lowering LDL-cholesterol levels is a primary therapy to reduce CVD risk.

HMG-CoA reductase inhibitors, better known as statins, are the most prescribed class of drugs worldwide and are widely used in the prevention of CVD. Statin therapy has been proven to lower LDL-cholesterol levels up to 55% [5] and reduce the incidence of CVD events by 20-30% [6]. Despite the clinical effectiveness of statins, there is large variability in clinical response to statin therapy. For example, within the PROSPER trial, 13% of the subjects allocated to statin treatment did not reach >10% LDL-cholesterol lowering after 36 months of pravastatin treatment [7]. In recent years, several studies have shown that genetic factors may influence the inter-individual variation in response to statin therapy [8;9].

Pharmacogenetic studies are used to assess whether genetic variation contributes to the variability in clinical response to drug therapy. The aim of pharmacogenetic studies is to develop better patient- or disease-specific health care [10]. In the past, pharmacogenetic studies were mainly performed by investigating only one genetic variant at a time. These candidate gene studies focused on genes hypothesized to be involved in the disease or drug response. Nowadays more and more genome-wide associations studies (GWAS) are performed. Unlike candidate gene approaches, GWAS can link multiple genetic variants with no *a priori* assumptions, thereby facilitating new discoveries [11]. Pharmacogenetic studies with regard to statin therapy

have mainly focused on the lipid lowering response to statins. Several studies have shown that genetic variation within the *APOE* and *LPA* genes are associated with the level of LDL-cholesterol lowering after statin therapy [12-14]. Less is known about genetic variation associated with variability in clinical event reduction and side effects in response to statin therapy.

Besides improving CVD treatment by identifying genetic variants associated with the variability in statin response, another option would be to develop new treatment options. One of the promising new LDL-cholesterol lowering drugs are the proprotein convertase subtilisin-like/kexin type 9 (PCSK9) inhibitors. PCSK9 is a protein involved in the LDL-cholesterol metabolism by promoting the degradation of LDL receptors [15]. Several phase I and II clinical trials have shown that inhibiting PCSK9 by monoclonal antibodies is very effective in lowering LDL-cholesterol levels [16]. Phase III trials including more participants followed over a longer period are currently ongoing to assess the effects of the inhibitors on CVD and other adverse events [16].

The aim of the PROSPER trial was to investigate the effectiveness of pravastatin in the elderly. Pravastatin reduced the LDL-cholesterol levels by 34% and reduced the incidence of CVD by 15%, and was thereby shown to be effective even in the elderly [7]. However, the association between LDL-cholesterol and CVD and mortality risk at older ages is controversial. Observational studies including elderly participants have shown no or inverse associations [17]. This might be explained by the observation that at old age, LDL-cholesterol levels in plasma may not reflect their life-time LDL-cholesterol levels due to comorbidities [18]. The use of genetic variants, associated with LDL-cholesterol levels, provides a possibility to investigate the association between LDL-cholesterol and CVD and mortality in the elderly free of biases.

Outline of this thesis

In this thesis, several of the issues described above are addressed, with the aim to give more insight in the (pharmaco) genetics of lipid metabolism in cardiovascular disease. In the first four chapters the focus will be on the pharmacogenetics of statin therapy. In **chapter 2** an extensive overview is given of the literature on candidate gene studies and GWAS investigating the pharmacogenetic effects on statin therapy. In **chapter 3** we describe the PHArmacogenetic study of Statins in the Elderly at risk (PHASE), the GWAS performed in the PROSPER study. This GWAS is used in pharmacogenetic studies within PROSPER to identify genetic variation responsible for individual variation in drug response to pravastatin. In addition, we used the GWAS on baseline LDL-cholesterol levels to validate the use of the GWAS for future genetic

studies. In **chapter 4** the first results of the analyses performed for the Genomic Investigation of Statin Therapy (GIST) consortium are shown. Collaboration in large consortia is needed to increase the power of pharmacogenetic analysis. In this chapter we present the results of the pharmacogenetic meta-analysis of GWAS of LDL-cholesterol response to statins, with the aim of determining whether additional loci influence LDL-cholesterol response to statins. In **chapter 5** we performed a GWAS in the PROSPER/PHASE study to investigate whether there are genetic variants associated with a clinically meaningful differential event reduction by pravastatin treatment.

In **chapter 6** we tried to distinguish between non-responders and non-adherers to statin therapy in the PROSPER study. In pharmacogenetic studies, genetic variation in non-responders and high-responders are compared with the goal to identify genetic loci associated with the inter-individual variation in drug response. However, in this context it is an important question whether the non-responders are true non-responders or whether they are actually non-adherent. In **chapter 7** we shortly discuss the usage of statins worldwide and whether there are indications that they would work differently in different parts of the world.

In **chapter 8** a genetic risk score was created based on genetic variants associated with variation in LDL-cholesterol levels. We used this genetic risk score to assess the unconfounded associations between LDL-cholesterol and mortality and longevity in three Dutch cohorts.

Since LDL-cholesterol is an important risk factor of CVD, even at old age, the aim is to develop new treatment approaches to decrease LDL-cholesterol levels. One of these are the PCSK9 inhibitors. Since it is not known whether these inhibitors will have an influence on cognitive performance or clinical events, we assessed in **chapter 9** the relation between genetic variants within the *PCSK9* gene and cognitive function and non-cardiovascular events in the participants of the PROSPER study.

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CHAPTER 2

Pharmacogenetics of statins: achievements, whole-genome analyses and future perspectives

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Abstract

Statins are the most commonly prescribed class of drug worldwide and therapy is highly effective in reducing low-density lipoprotein cholesterol levels and cardiovascular events. However, there is large variability in clinical response to statin treatment. Recent research provides evidence that genetic variation contributes to this variable response to statin treatment. Until recently, pharmacogenetic studies have used mainly candidate gene approaches to investigate these effects. Since candidate gene studies explain only a small part of the observed variation and results have often been inconsistent, genome-wide association (GWA) studies may be a better approach. In this paper the most important candidate gene studies and the first published GWA studies assessing statin response are discussed. Moreover, we describe the PHASE study, an EU-funded GWA study that will investigate the genetic variation responsible for the variation in response to pravastatin in a large randomized clinical trial.

Introduction

Cardiovascular disease is the leading cause of death in industrialized countries [1]. The HMG-CoA reductase (HMGCR) inhibitors, also known as statins, are the most prescribed class of drug worldwide and are widely used in the prevention of cardiovascular disease. Statin therapy is generally associated with a low-density lipoprotein (LDL) cholesterol lowering up to 55% [2] and a reduction of cardiovascular events by 20-30% [3]. Despite the clinical effectiveness of statins, there is large variability in clinical response to statin treatment. For example, within the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) trial, a large randomized clinical trial assessing the effectiveness of pravastatin in elderly, compliance with study medication was high, yet 13.3% of the subjects allocated to pravastatin did not reach 10% LDL-cholesterol lowering after 36 months of pravastatin treatment [4]. Many studies from the past years provide evidence that genetic factors contribute to this inter-individual variation in drug response [5;6]. The genetic variation associated with lipid lowering in response to statin therapy has been investigated mainly by previous pharmacogenetic studies. Relatively little is known about the genetic variation associated with variability in clinical events and side effects in response to statin therapy. The aim of this paper is to give an overview of the literature on candidate gene studies and the more recent performed genome-wide association (GWA) studies of pharmacogenetics of statins and to introduce the PHArmacogenetic study of Statin in the Elderly at risk (PHASE).

Pharmacogenetic studies investigating variable lipid-lowering response after statin therapy

Pharmacogenetic studies are performed to assess whether genetic variation accounts for the variability in clinical response to drug therapy. Meaningful candidate genes for investigating statin response are genes that belong to lipid metabolism, inflammation, thrombosis, and endothelial function as well as pharmacodynamic target genes, disease-modifying genes, and genes involved in uptake, distribution and metabolism of statins (see Figure 1 for the pharmacodynamic and pharmacokinetic statin pathways) [5]. More than 40 candidate genes have been described with respect to the variable effect of statins in lipid-lowering abilities, and the variable effect on the risk of clinical endpoints including myocardial infarctions and cardiovascular death [6].

HMGCR is the rate limiting enzyme in cholesterol synthesis. Statins are competitive inhibitors of HMGCR and therefore this gene is an interesting target for pharmacogenetic studies. The largest reported pharmacogenetic study investigating

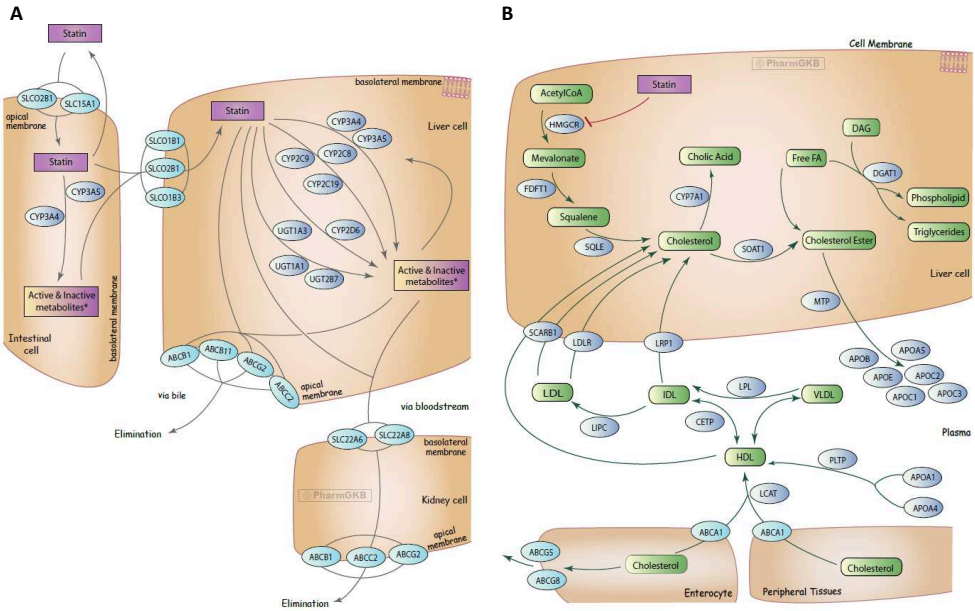

Figure 1. Pharmacokinetic (a) and pharmacodynamics (b) pathways of statins
Abbreviations: FA: Fatty acid; HDL: High-density lipoprotein; IDL: Intermediate-density lipoprotein; LDL: Low-density lipoprotein; VLDL: Very low-density lipoprotein.
Reproduced from McDonagh *et al*. [55]. Used with permission of PharmGKB and Stanford University.

genetic variation in various candidate genes was performed in 1536 participants of the PRINCE study [7]. One hundred and forty eight single nucleotide polymorphisms (SNPs) in 10 candidate genes known to be involved in cholesterol synthesis and statin metabolism were investigated. After correcting for multiple testing, two common intronic SNPs (chromosome 5 position 74726928 and 74739571, Human genome July 2003 UCSC version hg16, based on build 34) in the *HMGCR* gene were significantly associated with a reduced response to pravastatin therapy. Those SNPs were tightly linked (linkage disequilibrium r^2=0.90) and the results of the two SNPs were equivalent. Carriers of one copy of the minor allele of one of the SNPs had a 22% less relative reduction in total cholesterol levels and 19% less relative reduction in LDL-cholesterol compared with participants homozygous for the major allele of one of the SNPs. Interestingly, no differences in baseline lipid levels were seen between the genotypes.

Genetic variation of *APOE*, and in particular the ε2/ε3/ε4 variants (coded by rs7412 and rs429358), have been investigated extensively [8]. ApoE has various roles in lipid and lipoprotein metabolism and thus a clear impact on plasma lipid and lipoprotein levels. It has been shown in many studies that the *APOE* ε4 and ε2 alleles associate

with higher and lower concentrations of total cholesterol, LDL-cholesterol and ApoB, respectively, compared with the ε3 allele [9-11]. The results of studies on *APOE* SNPs and statin therapy response are equivocal, which has been summarized in a review by Nieminen *et al.* [8]. Several studies report less effect of statins in ε4 carriers for lowering cholesterol levels, compared with ε3 carriers, whereas carriers of the ε2 allele have a larger reduction of cholesterol levels during statin therapy compared with ε3 carriers. Nevertheless, several studies found no significant associations for *APOE* SNPs and lipid levels during statin therapy [8]. A recent meta-analysis did also not confirm the association between *APOE* SNPs and lipid response during statin therapy [12].

Other pharmacogenetic studies have investigated genetic variation in LDL-cholesterol related candidate genes, for example the LDL-receptor gene [13]. However, most of the results from studies investigating changes in lipid levels and cardiovascular event responses are difficult to interpret, because of strong influences of the genetic variation on baseline lipid levels [5]. Also several genes have been investigated for their pharmacokinetic and dynamic influences on statins. Two of those genes are *SLCO1B1*, the gene encoding the solute carrier organic anion transporter family member 1B1 (OATP1B1) influx, and ATP-binding cassette sub-family B member 1 (ABCB1) efflux transporter. The group of Niemi *et al.* showed that *ABCB1* haplotypes (rs1045642, rs2032582 and rs1128503) affected the pharmacokinetics of the active acid forms of simvastatin and atorvastatin [14]. *In vivo* pharmacokinetic studies have shown associations between *SLCO1B1* variations and statin plasma concentrations. Those pharmacokinetic features could only be translated to cholesterol-lowering abilities in small *in vivo* pharmacodynamic studies, results from larger studies are contradictory [15]. Other genes involved in the metabolism of statins are for example *CYP3A4* and *ABCG2,* but research has not been able to produce definitive results to show the possible role of these genes in the pharmacogenetics of statins [16].

Pharmacogenetic studies investigating variation in clinical events after statin therapy

Kinesin-like protein 6 (KIF6) is a member of the molecular motor superfamily involved in intracellular transport of several important molecules, including mRNA [17]. Several studies have shown an association between the Trp719Arg (rs20455) SNP in the *KIF6* gene and coronary heart disease [18-23]. Furthermore, analyses in four large clinical trials have shown a substantially increased benefit of statin therapy in carriers of this SNP compared with non-carriers [18;19;24]. In the WOSCOPS study, a primary prevention statin trial, the absolute risk reduction of coronary heart disease by statin therapy was 5.5% in carriers of the SNP compared with 0.1% in noncarriers

[19]. In the secondary prevention trials PROSPER, CARE, and PROVE IT - TIMI 22, the absolute risk reduction by statin therapy ranged from 5% to 10% in carriers of the SNP compared with 0.4% to 1.2% in noncarriers. The endpoints of interest in those studies were respectively: coronary events, myocardial infarction, and death or major cardiovascular events [18;19;24]. However, those results are equivocal; an accompanying editorial in the *Journal of the American College of Cardiology* expressed their doubts about the validity of those studies [25]. Moreover, a recent meta-analysis of 19 case-control studies (in total 17,000 coronary artery disease (CAD) cases and 39,369 controls) reported no association between the *KIF6* SNP and the risk of clinical CAD [17]. Furthermore, within the 18,348 participants from the HPS study, the *KIF6* SNP was not associated with the risk of incident vascular events among placebo treated participants, and reductions in the risk of vascular events during statin therapy were similar across *KIF6* genotypes [26].

Another gene that has been analyzed comprehensively is the *CETP* gene. *CETP* is involved in cholesterol metabolism by transporting cholesteryl ester back into the liver and functions to transport triglycerides from LDL and very low-density to high-density lipoprotein (HDL) cholesterol [27]. The most investigated SNP in *CETP* is the *Taq*1B variant (rs708272), a SNP in the first intron of the *CETP* gene. Initial studies associated the B2B2 genotype of the *CETP* gene with lower CETP levels [28], higher HDL-cholesterol levels, and a lower risk of progression of CAD, compared with the B1B1 *CETP* genotype [29-32]. However, when patients with the B1B1 genotype were treated with statins, they showed a lower progression of CAD compared with B2B2 carriers. In addition, long-term results from the REGRESS study, the first study to report the possible pharmacogenetic interactions between the *CETP* SNP and statin treatment [33], demonstrated significantly higher 10-year mortality in statin-treated male B2 carriers, compared with carriers of the B1B1 genotype [34]. Therefore, although untreated B2B2 patients have a lower risk of CAD progression, statin treatment is more beneficial in patients with the B1B1 genotype, denying the initial advantage of the B2 allele in CAD. A large meta-analysis including 13,677 subjects confirmed the association between the *Taq*1B SNP and HDL-cholesterol levels and the risk of CAD, but the interaction between the SNP and statin therapy could not be confirmed [35].

SNPs in the *APOE* gene have also been assessed in the relation with progression of coronary heart disease during statin therapy[8]. Gerdes *et al.* analyzed data of 5.5 years of follow-up from 966 Danish and Finnish myocardial infarction survivors enrolled in the Scandinavian Simvastatin Survival Study (4S) [36]. Carriers of the *APOE* ε4 allele had nearly twofold higher mortality compared with noncarriers of the ε4

allele during simvastatin therapy. However, the results found in the 4S trial are equivocal; analyses in almost 8000 participants from the Rotterdam Study and in 815 men in the REGRESS study could not confirm the pharmacogenetic effect of statins on cardiovascular endpoints [37;38].

Two another genes investigated in relation with statin therapy and clinical events are *SLCO1B1* and *ABCB1*. Peters *et al.* tested 24 tagging SNPs in the two genes in 668 cases with myocardial infarction and 1217 controls from the population-based PHARMO study [39]. They found two SNPs within *ABCB1* (rs3789244 and rs1922242) to interact significantly with statin therapy. In addition, they observed a non-significant interaction between the *SLCO1B1*1A* haplotype and statin treatment; odds ratio (OR) homozygote carriers 0.49 (95% CI 0.34-0.79) compared with 0.31 (95% CI 0.24-0.41) for heterozygous or noncarriers of the **1A* allele [39].

GWA studies

As shown in the previous paragraphs, various studies have assessed the association between genetic polymorphisms and response to statin therapy. At least two reviews have given an elaborate overview of the pharmacogenetic candidate genes and their genotype effects related to statin therapy [5;6]. Table 1 shows a brief overview of the most important investigated candidate genes in their relation to efficiency and clinical effectiveness after statin therapy. Based on these data, candidate genes regulating pharmacokinetic and pharmacodynamic properties of statins appear to be the most promising target genes (see figure 1). Although the genetic variation in these pharmacokinetic and pharmacodynamic pathways has received much attention over the past years, it only explains a small part of the observed variation, and the results are often inconsistent. Therefore, it is now important to investigate which other genetic pathways are responsible for the remaining genetic variation in statin response.

GWA studies are another approach to investigate pharmacogenetic effects. Unlike candidate gene approaches, GWA studies can link multiple SNPs to drug response with no *a priori* assumptions, thereby facilitating new discoveries. At present, only three GWA studies investigating genetic variants and variation in response to statin therapy have been published, focusing on lipid lowering and adverse effects of statin therapy [40-42]. The first published GWA study on statin response was performed in the TNT study. This study used a combination of a genome-wide and candidate gene approach. Using only the GWA study in 1984 individuals, no SNPs were genome-wide significantly associated with statin response. However, by analyzing the candidate genes in the genotyped participants, they found the SNP rs7412 in the *APOE* gene

Table 1. Overview of a selection of the genes investigated for associations with statin treatment and several outcomes

Gene	Main outcome parameter of the study	Statin	Study type	Results	Ref
HMGCR	Plasma lipids	Pravastatin, simvastatin, atorvastatin	Candidate gene	SNPs associated with LDLC and TC lowering	7,40,56
APOE	Plasma lipids	Pravastatin	Candidate gene	No association	7
	Plasma lipids, coronary events	Atorvastatin	Candidate gene and GWAS	SNP associated with LDLC response but not with clinical events	40
	Plasma lipids	Various	Meta-analysis	No association	12
	Death or major coronary event	Simvastatin	Candidate gene	SNP associated with mortality but not with major coronary events	36
	Plasma lipids, coronary angiography	Pravastatin	Candidate gene	SNP associated with lipids but not with angiography	37
	MI, stroke and mortality	Various	Candidate gene	No association	38
ABCB1	Pharmacokinetics	Simvastatin, atorvastatin	Candidate gene, in vivo	SNPs affect the pharmacokinetics	14
	Myocardial infarction	Various	Candidate gene	Interaction between SNPs and statin treatment	39
SLCO1B1	Pharmacokinetics	Various	Candidate gene, in vivo	SNPs affect the pharmacokinetics	15
	Myopathy	Simvastatin	GWAS	SNP associated with risk for myopathy	41
	Myopathy	Simvastatin, atorvastatin	Candidate gene	SNP associated with myopathy in simva users, but not in atorva users	43
	Myocardial infarction	Various	Candidate gene	Non-significant interaction between haplotype and statin treatment	39
KIF6	Coronary events	Pravastatin, atorvastatin	Candidate gene	Association between SNP and events	18,19,24
	Coronary events	Simvastatin	Candidate gene	No association	26
CETP	Lipid lowering	Pravastatin	Candidate gene	No association	7
	CAD	Pravastatin	Candidate gene	SNP associated with CAD progression	29
	CV events	Various	Candidate gene	SNP associated with risk of CV events	57
	CV events	Pravastatin	Candidate gene, meta-analysis	No treatment interaction	30,35
	Mortality	Pravastatin	Candidate gene	SNP associated with 10-yr mortality	34
CLMN	Plasma lipids	Simvastatin, pravastatin, atorvastatin	GWAS meta-analysis	SNP associated with TC lowering	42

Abbreviations: SNP, Single nucleotide polymorphism; LDLC, low-density lipoprotein cholesterol; TC, total cholesterol; GWAS, Genome-wide association study; MI, Myocardial infarction; CAD, coronary artery disease; CV, cardio vascular.

significantly associated with statin response ($p=3.65 \times 10^{-9}$). The rs7412 SNP was not present on the platform used for the GWA scan used in the TNT study and was not in linkage disequilibrium with any of the SNPs in the GWA scan [40].

The second published GWA study on statin response was a meta-analysis performed in approximately 4,000 subjects from three statin trials; the CAP trial, the PRINCE study, and the TNT study. An association between the gene *CLMN*, encoding calmin, and the reduction in total cholesterol levels after statin treatment was observed. The function of calmin is unknown and has not been implicated in cholesterol metabolism before. The combined analysis of these three studies found an 84% posterior probability that the *CLMN* SNP (rs8014194) was genuinely associated with statin-mediated change in total cholesterol ($p=1.9 \times 10^{-8}$). On average across the three studies, carriers with two copies of the minor allele of rs8014194 had a 3% lower total cholesterol reduction compared with noncarriers [42]. Nevertheless, further (functional) studies are needed to replicate this finding and explore the function of calmin.

The third published GWA study investigated genetic variation in relation with statin-induced myopathy, an adverse side effect of statin therapy. This GWA study included 85 subjects with definite or incipient myopathy and 90 controls, all taking 80 mg simvastatin daily. The SNP rs4363657 located within the *SLCO1B1* gene, which encoded the polypeptide OATP1B1, which mediates the hepatic uptake of most statins, was found to be associated with myopathy ($p=4.1 \times 10^{-9}$). The noncoding rs4363657 SNP was in nearly complete linkage disequilibrium with the non-synonymous rs4149056 SNP (Val174Ala; $r^2=0.97$), which was also located within the *SLCO1B1* gene and has been linked to statin metabolism. The OR for myopathy was 4.3 per copy of the rs4363657 C allele (minor allele frequency: 0.13) and 17.4 in homozygote carriers of the CC variant compared with homozygote carriers of the common variant (TT) [41]. This is the strongest effect found until now from a pharmacogenetic effect in relation to the response to statin therapy. Brunham *et al.* have performed a study aiming to replicate the association of rs4149056 and statin-induced myopathy is a cohort of patients using various statin types [43]. In this small study, including 25 cases of severe statin-induced myopathy and 83 controls, the SNP was not associated with myopathy in the complete group. However, stratifying patients by statin type, the SNP was significantly associated with myopathy in simvastatin users (OR: 3.2, p=0.042), but not in atorvastatin treated patients (OR: 4.5, p=0.48). These different results between different statin types indicate the presence of possible statin type-specific pharmacogenetic effects.

Pharmacogenetic study of Statin in the Elderly at risk, a large GWA study

Results of pharmacogenetic candidate gene studies are often inconsistent and explain only a small part of the observed variation in statin response. Furthermore, GWA studies enable detection of novel and less obvious genes. Since there are currently only three GWA studies on statin response published with only very low subject numbers, we have initiated the PHArmacogenetic study of Statin in the Elderly at risk (PHASE) [44]. PHASE is a EU sponsored GWA study in the participants of the PROSPER study [4] investigating the genetic variation responsible for the individual variation in drug response. The PROSPER study provides a good population to study pharmacogenetics. First, PROSPER is an investigator-driven, prospective multi-national randomized placebo-controlled trial including 5,804 subjects aged 70-82 years at baseline of whom more than 50% was female. Plasma levels of LDL-cholesterol as well as other levels of plasma lipoproteins were measured at baseline and prospectively during follow-up for a mean of 3.2 years (range 2.8-4.0). Second, within the PHASE study 557,192 SNPs in 5,244 subjects are available for analysis, and to maximize the availability of genetic data and coverage of the genome those SNPs have been imputed up to 2.5 million SNPs. A GWA study for LDL-cholesterol was used as proof-of-principle analysis in the PROSPER/PHASE study. With this GWA study five of the previously found genetic associations with LDL-cholesterol were confirmed and shows that we are able to detect genetic effects within the elderly participants of PROSPER [44].

The large number of statistical tests performed in a GWA analysis requires large sample sizes to provide adequate statistical power to detect small effect sizes. For this purpose it is necessary to cooperate with other studies. To investigate genetic loci affecting statin response and adverse effects the PHASE study is involved in the Genomic Investigation of Statin Therapy (GIST) consortium. GIST is a large international consortium formed to conduct a combined meta-analysis of GWA and replication studies, including several randomized controlled trials (RCT) of statin therapy and several non-trial cohorts of statin recipients with GWA data. Most of the large RCTs are participating in the GIST consortium, including the PROSPER/PHASE study, the CAP trial, the PRINCE study, the TNT study, the CARDS study [45], and the ASCOT trial [46]. Together those studies provide data of approximately 10,000 statin treated subjects. The non-trial cohorts involved in the GIST consortium are the cohorts of the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium [47]. The studies from CHARGE are supplemented with the MESA study [48], the HABC study [49], the HVH study [50], the GoDARTs study [51], and the biobank of the Vanderbilt University (BioVu) [52]. With those studies participating in GIST there is observational data available of

approximately 10,000 statin treated subjects. The first plan for the GIST consortium is to conduct a meta-analysis of GWA studies and replication studies to identify novel loci influencing statin response. For this aim every participating study will perform their own analysis assuming an additive genetic model. To overcome the less certainty of imputed SNPs, regression analysis onto expected allele dosages will be performed. The meta-analysis will be performed with the random effects model. To correct for multiple testing, we will use a p-value threshold of $5.0x10^{-8}$ for statistical significance. To conclude, with the GIST consortium we have data of approximately 20,000 statin treated subjects, which gives the opportunity to assess the genetic variation responsible for the variable response to statin treatment in a large consortium.

Future perspective

Over previous years, substantive effort has been made in investigating the pharmacogenetics of the variable response to statin treatment. With the GWA analysis performed in the PHASE study and the meta-analysis in the GIST consortium we hope to identify novel genes and pathways involved in the variation in statin response. Expanding the knowledge about the genes and pathways associated with the variation in statin response might lead to substantial improvements in the use of cardiovascular drug therapy, through selection of the most appropriate drug therapy based on an individual's genetic make-up [53;54]. With the results of the PHASE study and the GIST consortium we aim to identify nonresponders or subjects who will experience adverse effects by their genetic variation.

However, with the GWAS study performed in the PROSPER/PHASE study and the GIST consortium only common variants associated with statin response will be detected. Within the PROSPER/PHASE study we will perform an exome sequencing study to identify also rare variants associated with statin response. High responders to statin therapy will be compared with low or nonresponders in order to find the biological pathways involved in pharmacogenetics of statin therapy. Moreover, epigenetic studies will be executed to investigate the epigenetic mechanisms involved in the interindividual variation in response to statin treatment. All genetic or epigenetic variation that will be identified by these studies will be further tested in a clinical setting to investigate their use in clinical practice.

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CHAPTER 3

Replication of LDL GWAS hits in PROSPER/PHASE as validation for future (pharmaco)genetic analyses

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Abstract

The PHArmacogenetic study of Statins in the Elderly at risk (PHASE) is a genome wide association study in the PROspective Study of Pravastatin in the Elderly at risk for vascular disease (PROSPER) that investigates the genetic variation responsible for the individual variation in drug response to pravastatin. Statins lower LDL cholesterol in general by 30%, however not in all subjects. Moreover, clinical response is highly variable and adverse effects occur in a minority of patients. In this report we first describe the rationale of the PROSPER/PHASE project and second show that the PROSPER/PHASE study can be used to study pharmacogenetics in the elderly. The genome wide association study (GWAS) was conducted using the Illumina 660K-Quad beadchips following manufacturer's instructions. After a stringent quality control 557,192 SNPs in 5,244 subjects were available for analysis. To maximize the availability of genetic data and coverage of the genome, imputation up to 2.5 million autosomal CEPH HapMap SNPs was performed with MACH imputation software. The GWAS for LDL cholesterol is assessed with an additive linear regression model in PROBABEL software, adjusted for age, sex, and country of origin to account for population stratification. Forty-two SNPs reached the GWAS significant threshold of p=5.0e-08 in 5 genomic loci (*APOE*/*APOC1*; *LDLR*; *FADS2*/*FEN1*; *HMGCR*; *PSRC1*/*CELSR5*). The top SNP (rs445925, chromosome 19) with a p-value of p=2.8e-30 is located within the *APOC1* gene and near the *APOE* gene. The second top SNP (rs6511720, chromosome 19) with a p-value of p=5.22e-15 is located within the *LDLR* gene. All 5 genomic loci were previously associated with LDL cholesterol levels, no novel loci were identified. Replication in WOSCOPS and CARE confirmed our results. With the GWAS in the PROSPER/PHASE study we confirm the previously found genetic associations with LDL cholesterol levels. With this proof-of-principle study we show that the PROSPER/PHASE study can be used to investigate genetic associations in a similar way to population based studies. The next step of the PROSPER/PHASE study is to identify the genetic variation responsible for the variation in LDL cholesterol lowering in response to statin treatment in collaboration with other large trials.

Introduction

Cardiovascular disease is the leading cause of death in industrialized countries at old age. Advancing age is one of the most important risk factors for cardiovascular disease [1]. With the rising number of elderly people in our society cardiovascular disease has a major impact on healthcare [2]. The prevention of cardiovascular disease is critically dependent on lipid lowering therapy including the 3-hydroxymethyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins). Statins are the most prescribed class of drugs worldwide and therapy is generally associated with a reduction of cardiovascular events by 20-30%. However, clinical response is highly variable and adverse effects occur in a minority of patients [3]. Recent research provides evidence that genetic variation contributes importantly to this variable drug response [4].

Pharmacogenomics focuses on unraveling the genetic determinants of such variable drug responses, both in intended, beneficial effects and unintended, adverse effects [5]. Therefore, we here present the PHArmacogenetic study of Statin in the Elderly at risk (PHASE) a genome wide association study (GWAS) in the PROspective Study of Pravastatin in the Elderly at Risk for vascular disease (PROSPER)[6] investigating the genetic variation responsible for the individual variation in drug response funded by the European Union's Seventh Framework Programme. To validate the GWAS performed in the PHASE study, we executed a proof-of-principle study to investigate the underlying genetic variation in LDL cholesterol levels.

Recent GWA studies have identified several new loci that influence circulating levels of blood lipids with around 95 loci showing statistical associations with circulating total cholesterol levels, HDL cholesterol, LDL cholesterol, and triglycerides [7]. These GWA studies are executed in population based studies with various age groups, however the elderly (age >75 years) are rarely represented in these studies. With this proof-of-principle study we provide a testing frame to show that the PROSPER/PHASE study has sufficient statistical power to find genome wide statistical significant associations in quantitative traits such as LDL cholesterol in an elderly population. We replicated our findings from the PROSPER/PHASE study in two independent cohorts to validate that our results contain no false positive findings.

Methods

Study population

PROSPER was an investigator-driven, prospective multi-national randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in the elderly [6;8]. Between December 1997 and May 1999, we screened and enrolled subjects in Scotland, Ireland, and the Netherlands. Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or were at increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5804 subjects, of whom more than 50% was female, were randomly assigned to pravastatin or placebo. Various clinical laboratory measurements were carried out like inflammatory markers (CRP and various cytokines) and other biochemical substrates (e.g. glucose, leptin) at baseline and during follow-up. The protocol of the PROSPER study meets the criteria of the Declaration of Helsinki and was approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from all participating subjects.

LDL cholesterol

Plasma lipids and lipoproteins were measured twice during the screening phase, i.e. at the beginning and end of the single-blind, placebo "run-in" phase according to the standardized Lipid Research Clinics protocol. Baseline LDL cholesterol levels were taken as the average of these 2 determinations prior to randomization to statin treatment. Total cholesterol (TC), HDL cholesterol, and triglycerides were assessed after an overnight fast, LDL cholesterol was calculated by the Friedewald formula, as previously described [8].

Genotyping

The genotyping was conducted using the Illumina 660-Quad beadchips following manufacturer's instructions. These beadchips contain 657,366 single nucleotide polymorphism (SNP) and copy number variants (CNV) probes. After genotyping, samples and genetic markers were subjected to a stringent quality control protocol. From the 5763 samples with DNA available that underwent genotyping, 519 samples (9%) were excluded during the quality control (figure 1). Excluded were 18 duplicated samples, 219 samples with a call rate <97.5%, 11 samples with an excess for heterozygosity, 40 samples of non-caucasian origin, 170 samples with familiar relationships (IBD>0.35), and 61 samples with a gender mismatch. From the 657,366 probes on the beadchips, 95,876 probes were filtered based on CNV intensity. Moreover, 4,298 SNPs were excluded with a call rate < 95%, leaving us with 557,192

SNPs for analysis. To maximize the availability of genetic data and coverage of the genome, imputation up to 2.5 million autosomal CEPH HapMap SNPs was performed with MACH imputation software based on the HapMap built II release 23. To assess accuracy of the imputed genotypes, we compared the imputation output with SNPs that had been previously genotyped on other platforms.

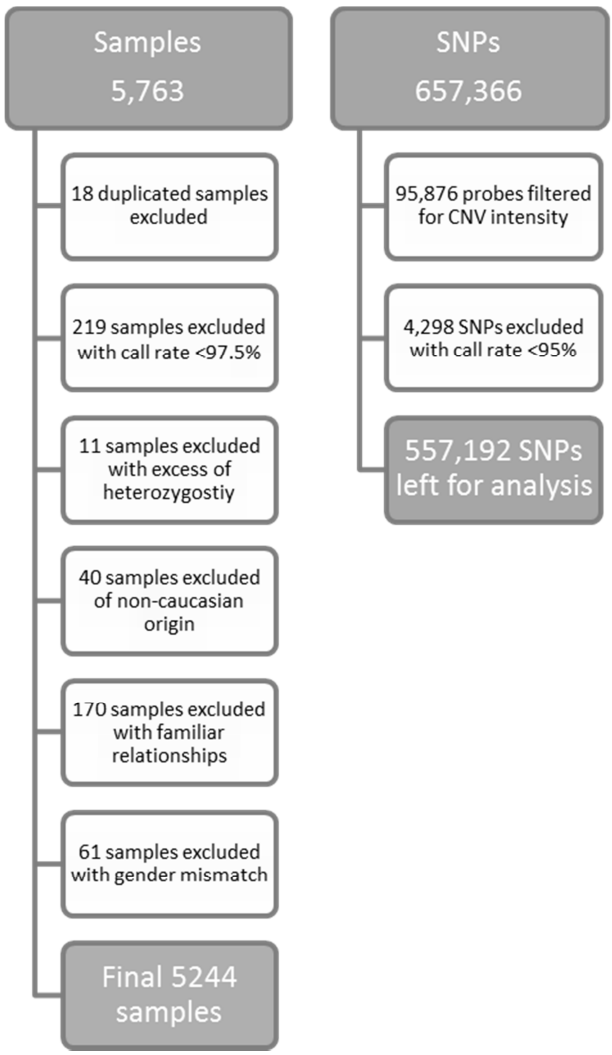

Figure 1. Flow chart of the Quality Control of the PROSPER/PHASE study

Statistical Analysis

Genome wide association analysis was performed with PROBABEL software specialized in genetic association analysis with imputed data taking the probability of the genotype into account (http://www.genabel.org/). With analyzing imputed genotypes, the observed allele count is replaced by the imputation's estimated dosage. For the continuous trait, baseline LDL cholesterol levels, an additive linear regression model was used to assess estimates and standard errors. The model was adjusted for sex and age, and country to correct for the within-study population structure. Standard errors for the regression estimates were calculated with model-robust methods. The analysis of 2.5 million SNPs at once poses a multiple testing problem. After the use of a Bonferroni correction, the threshold for genome wide significant results was set at 5.0e-08.

Replication

Associations with a genome-wide significant p-value of 5.0e-08 were replicated in two independent cohorts, the West of Scotland Coronary Prevention Study (WOSCOPS) [9] and the Cholesterol and Recurrent Events (CARE) trial [10]. The WOSCOPS study was a double blind randomized placebo-controlled clinical trial in which 6595 men (age range 45-64 years)with hypercholesterolemia and no history of myocardial infarction were treated with 40 mg pravastatin (N=3302) or placebo (N=3293). GWAS data and baseline LDL cholesterol levels were available for 431 subjects. The CARE study was a double blind randomized placebo-controlled clinical trial in which 4159 patients (age range 21-75 years) were treated with 40 mg pravastatin (N=2081) or placebo (N=2078). GWAS data and baseline LDL cholesterol levels were available for 751 subjects. The significance level for the replication SNPs was set at p-value < 0.05.

Results

Table 1 shows the baseline characteristics of the subjects participating in the PROSPER and the PROSPER/PHASE study. This table shows that the genotyped subjects in the PROSPER/PHASE study are representative of the total study population of the PROSPER study, since no major discrepancies exist between the two study sets. The mean age of all subjects at study entry was 75.3 years and about 50% of the participants were female.

In Figure 2 the QQ-plot of the genome-wide association study with baseline LDL levels within the PROSPER/PHASE study is shown. In this plot it is shown that no genomic inflation has occurred in this analyses (lambda=1.077) and that population stratification is sufficiently controlled for. In figure 3 the results of the genome-wide

Table 1. Baseline characteristics of the PROSPER/PHASE study

	PROSPER study (n=5804)	PROSPER/PHASE study (n=5244)
Continuous variables (mean, SD)		
Age (years)	75.3 (3.3)	75.3 (3.4)
Education (years)	15.1 (2.0)	15.1 (2.0)
Systolic blood pressure (mmHg)	154.7 (21.8)	154.6 (21.9)
Diastolic blood pressure (mmHg)	83.8 (11.5)	83.7 (11.4)
Height (cm)	165.2 (9.4)	165.2 (9.4)
Weight (kg)	73.4 (13.4)	73.3 (13.4)
Body mass index (kg/m^2)	26.8 (4.2)	26.8 (4.2)
Total cholesterol (mmol/L)	5.7 (0.9)	5.7 (0.9)
LDL cholesterol (mmol/L)	3.8 (0.8)	3.8 (0.8)
HDL cholesterol (mmol/L)	1.3 (0.3)	1.3 (0.4)
Triglycerides (mmol/L)	1.5 (0.7)	1.5 (0.7)
Categorical variables (n, %)		
Males	2804 (48.3)	2524 (48.1)
Current smoker	1558 (26.8)	1392 (26.5)
History of diabetes	623 (10.7)	544 (10.4)
History of hypertension	3592 (61.9)	3257 (62.1)
History of angina	1559 (26.9)	1424 (27.2)
History of claudication	390 (6.7)	354 (6.8)
History of myocardial infarction	776 (13.4)	708 (13.5)
History of stroke or TIA	649 (11.2)	586 (11.2)
History of vascular disease*	2565 (44.2)	2336 (44.5)

*Any of stable angina, intermittent claudication, stroke, transient ischemic attack, myocardial infarction, peripheral artery disease surgery, or amputation for vascular disease more than 6 months before study entry.

Table 2. Genomic loci with a genome wide significant p-value <= 5.0e-08 in the association with baseline LDL cholesterol levels

Chr.	Gene	Nr of SNPs	Top SNP	Variant	MAF	Beta	SE	p-value	Ref*
19	APOE/APOC1	17	rs445925	G>A	0.11	-0.33	0.03	2.8e-30	7;11-14;18;19
19	LDLR	5	rs6511720	G>T	0.13	-0.19	0.02	5.2e-15	7;11;13;14;19
5	HMGCR	5	rs258494	G>C	0.38	0.10	0.02	1.3e-09	7;11;13;14;19
11	FADS2/FEN1	14	rs174541	C>T	0.38	-0.10	0.02	1.1e-08	7;11;13;19
1	PSRC1/CELSR5	1	rs602633	G>T	0.23	-0.11	0.02	5.0e-08	7;11-14;16-19

Abbreviations: SNP, Single Nucleotide Polymorphism; Chr, Chromosome; MAF, minor allele frequency; SE, standard error. * A list of references in which the same loci were found.

Table 3. Replication of the 5 significant loci in the WOSCOPS trial and CARE study in association with baseline LDL cholesterol levels

			WOSCOPS (n=431)			CARE (n=751)		
SNP	**Gene**	**Chr.**	**beta**	**se**	**p-value**	**beta**	**se**	**p-value**
rs445925	APOE/ APOC1	19	0.07	0.05	0.164	-0.10	0.04	0.006
rs6511720	LDLR	19	-0.03	0.05	0.657	-0.03	0.03	0.411
rs258494*[1]	HMGCR	5	0.06	0.03	0.044	0.03	0.02	0.147
rs174541*[2]	FADS2/FEN1	11	-0.04	0.03	0.264	-0.03	0.02	0.134
rs602633*[3]	PSRC1/CELSR5	1	-0.09	0.04	0.026	-0.05	0.02	0.035

* A proxy for this SNP was used in both replication cohorts, for [1] the proxy SNP was rs7715806 with a r^2 of 0.93, for [2] the proxy SNP was rs174545 with a r^2 of 0.90, and for [3] the proxy SNP was rs660240 with a r^2 of 0.88.

Abbreviations: SNP, Single Nucleotide Polymorphism; Chr, Chromosome.

association study with baseline LDL cholesterol levels within the PROSPER/PHASE study are depicted in a Manhattan plot. Forty-two SNPs in five genomic loci, *APOE*/*APOC1*, *LDLR*, *FADS2*/*FEN1*, *HMGCR*, and *PSRC1*/*CELSR5*, reached the genome-wide significant p-value of 5.0e-08. In table 2 a summary of the five genomic loci and their corresponding SNPs is given. The top SNP (rs445925, Chr. 19) with a p-value of p=2.8e-30 is located within the *APOC1* gene and near the *APOE* gene. Sixteen other SNPs in the same genomic region were also found to be associated with LDL cholesterol levels. The second top SNP (rs6511720, Chr. 19) with a p-value of p=5.22e-15 is located within the *LDLR* gene. The three other genomic regions included the *HMGCR* (Chr.5), *FADS2*/*FEN1* (Chr. 11), *PSRC1*/*CELSR5* (Chr. 1) genes. All 5 genomic loci were previously found in association with LDL cholesterol levels and no novel loci were identified.

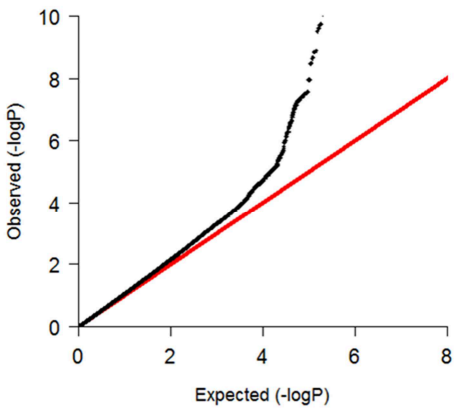

Figure 2. QQ-plot for the GWAS on baseline LDL cholesterol in the PROSPER/PHASE study.

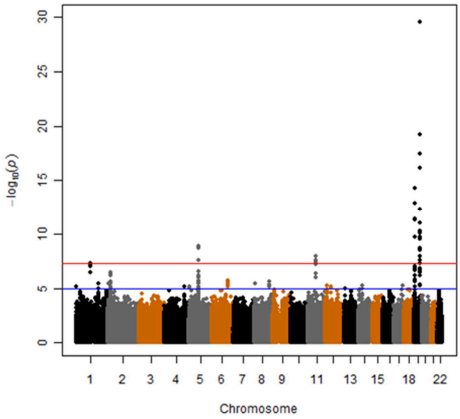

Figure 3. Manhattan plot for the GWAS on baseline LDL cholesterol in the PROSPER/PHASE study.

We replicated the positive associations with genome-wide significant p-values in two independent cohorts, the WOSCOPS study and the CARE trial (table 3). Of our five genomic loci that were significantly associated with baseline LDL cholesterol levels we selected the top SNP for replication in both replication cohorts. If the SNP was not genotyped in their GWAS analysis, we chose a proxy in high linkage disequilibrium (r2>0.5%) for that SNP. These SNPs were associated with baseline LDL levels before randomisation to statin treatment in both studies. Three out of the five loci (*APOE*/*APOC1*; *HMGCR*; *PSRC1*/*CELSR5*) replicated in one or two replication cohorts (p<0.05). The two other loci (*LDLR* and *FADS2*/*FEN1*) showed similar trends as shown in the discovery cohort, although they did not reach statistical significance (table 3).

Discussion

With this first proof-of principle study we show that the PROSPER/PHASE GWAS can confirm previously found genetic associations with LDL cholesterol levels. This proof-of-principle study indicates that the PROSPER/PHASE study is likely to be capable of detecting genomic regions responsible for the variation in various other quantitative traits. With almost 6000 samples in the PROSPER/PHASE study and access to various replication studies, the PROSPER/PHASE study can provide a good testing frame to identify the genetic variation responsible for the variation in LDL cholesterol lowering in response to statin treatment.

The main locus responsible for the person-to-person variation in LDL cholesterol levels is the chromosome 19 locus, which contains the *APOE*, *APOC1*, and *LDLR* genes. Other important loci included the *HMGCR* locus on chromosome 5, *FADS2*/*FEN1* locus on chromosome 11, and the *PSRC1*/*CELSR5* locus on chromosome 1. The five genomic loci that were associated with variation in LDL cholesterol levels in the PHASE GWAS study were all genomic regions that were previously reported with LDL cholesterol variation [7;11-19]. Three out of the five loci were replicated in the WOSCOPS study and the CARE trial. The *LDLR* and *FADS2*/*FEN1* loci were not replicated, however these loci were repeatedly found to be associated with LDL cholesterol levels in various other studies with large number of participants [7;11-14;16;19]. Moreover, both the WOSCOPS and CARE studies had genotype data available in a small number of subjects. Therefore, the lack of replication of these loci in WOSCOPS and CARE was most likely due to lack of statistical power. Finally, since we used in the replication studies a proxy SNP for some of the topSNPs, this may have diluted the effect.

Conclusions

With this proof-of-principle study we show that the PROSPER/PHASE study can be used to investigate genetic associations in a similar way to population based studies. Moreover, we can also assume from these results that the PROSPER/PHASE study is likely to have sufficient power to detect genome-wide significant hits with large effects for other quantitative traits. The next step of the PROSPER/PHASE study is to identify the genetic variation responsible for the variation in LDL cholesterol lowering in response to statin treatment.

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CHAPTER 4

Pharmacogenetic meta-analysis of genome-wide association studies of LDL cholesterol response to statins

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Abstract

Statins effectively lower LDL cholesterol levels in large studies and the observed inter-individual response variability may be partially explained by genetic variation. Here we perform a pharmacogenetic meta-analysis of genome-wide association studies (GWAS) in studies addressing the LDL cholesterol response to statins, including up to 18,596 statin-treated subjects. We validate the most promising signals in a further 22,318 statin recipients and identify two loci, *SORT1/CELSR2/PSRC1* and *SLCO1B1*, not previously identified in GWAS. Moreover, we confirm the previously described associations with *APOE* and *LPA*. Our findings advance the understanding of the pharmacogenetic architecture of statin response.

Introduction

The 3-hydroxymethyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also known as statins, are widely prescribed and are highly effective in the management and prevention of cardiovascular disease. Statin therapy results in a lowering of low-density lipoprotein cholesterol (LDL-C) levels by up to 55% [1] and a 20-30% reduction of cardiovascular events [2]. Despite the clinical efficacy of statins in a wide range of patients [2], inter-individual variability exists with regard to LDL-C lowering response as well as efficacy in reducing major cardiovascular events [3]. The suggestion that some of this variability may be due, in part, to common pharmacogenetic variation is supported by previous studies that have identified genetic variants associated with differential LDL-C response to statin therapy [4-6].

A small number of genome-wide association studies (GWAS) have previously identified loci associated with statin response on a genome-wide level. A GWAS in the JUPITER trial identified three genetic loci, *ABCG2* (rs2199936), *LPA* (rs10455872), and *APOE* (rs7412), that were associated with percentage LDL-C reduction following rosuvastatin therapy [7]. In the CARDS and ASCOT studies, single nucleotide polymorphisms (SNPs) at *LPA* (rs10455872) and *APOE* (rs445925 and rs4420638) were associated with LDL-C response to atorvastatin treatment [8]. A combined GWAS in three statin trials identified a SNP within *CLMN* (rs8014194) that is associated with the magnitude of statin-induced reduction in plasma cholesterol [9]. However, two other GWAS identified no genetic determinants of LDL-C response to statin therapy at a genome-wide significant level [6;10].

Based on these studies, as well as previous candidate gene studies [4;6], the only genetic variants that have been consistently identified to be associated with variation in LDL-C response to statin therapy, irrespective of statin formulation, are located at or nearby *APOE* and *LPA*. To determine whether additional loci may influence LDL-C response to statins, we formed the Genomic Investigation of Statin Therapy (GIST) consortium and conducted a pharmacogenetic meta-analysis using GWAS datasets from randomized controlled trials (RCTs) and observational studies. We identify two loci not previously identified in GWAS, *SORT1/CELSR2/*PSRC1 and *SLCO1B1.* In addition, we confirm the associations within the *APOE* and *LPA* genes. These findings will extend the knowledge of the Pharmacogenetic architecture of statin response.

Methods

Study populations

The meta-analysis was conducted in the Genomic Investigation of Statin Therapy (GIST) consortium, which includes data from eight randomized controlled statin trials (RCTs) and eleven prospective, population-based studies. The initial analysis (first stage) was performed in 8,421 statin treated subjects from six randomized controlled trials (ASCOT, CARDS, CAP, PRINCE, PROSPER, and TNT) and 10,175 statin treated subjects from ten observational studies (AGES, ARIC, BioVU, CHS, FHS, GoDARTS I, GoDARTS II, Health ABC, HVH, and MESA). Further investigation (second stage) was performed in 21,975 statin treated subjects from two randomized trials (HPS and JUPITER) and one observational study (Rotterdam Study). Six SNPs were additionally genotyped in the Scandinavian participants of the ASCOT study. The details of the first and second stage studies can be found in the Supplementary Table 1 and 2 and Supplementary Note 1 and 2.

Subjects

Response to statin treatment was studied in statin treated subjects only and not in those treated with placebo. Subjects included in the observational studies analysis should be treated with statins and have LDL-C measurements before and after start of statin treatment. Subjects of reported or suspected non-European ancestry were excluded. All participants gave written informed consent and the study was approved by all institutional ethic committees.

Outcome measurements

The response to statin treatment was defined as the difference between the natural log transformed on- and off-treatment LDL-C levels. The beta of the corresponding regression thus reflects the fraction of differential LDL lowering in carriers vs. non-carriers of the SNP. For observational studies the on-treatment LDL-C levels were taken into account for all kinds of prescribed statins, at any dosage, for any indication, and for at least four weeks prior to measurement. Characteristics of on- and off-treatment LDL-C levels and statins used in each study are shown in Supplementary Table 2. For each individual, at least one off-treatment LDL-C measurement and at least one on-treatment LDL-C measurement were required. When multiple on- or off-treatment measurements were available the mean of the cholesterol measurements was used. Subjects with missing on- or off-treatment measurements were excluded, with the exception of the GoDARTS cohorts for which missing off-treatment LDL-C levels were estimated using imputation methods

(Supplementary Note 2). In the HPS proportional LDL-C response was defined by the changes in natural log lipid levels from the screening visit prior to starting statin therapy to the randomization visit [6].

Genotyping and imputation

Genotyping, quality control, data cleaning and imputation was performed independently in each study using different genetic platforms and software as outlined in Supplementary Table 4. In all studies, genotyping was performed using Illumina, Affymetrix, or Perlegen genotyping arrays, and MACH, Impute, or BIMBAM software was used for imputation.

GWAS analysis

Each study independently performed the GWAS on the difference between natural log transformed on- and off-treatment LDL-C levels. To control for possible associations with off-treatment LDL-C levels, analyses were adjusted for the natural log transformed off-treatment LDL-C level. An additive genetic model was assumed and tested using a linear regression model. For imputed SNPs, regression analysis was performed onto expected allele dosage. Analyses were additionally adjusted for age, sex, and study specific covariates (e.g. ancestry PCs or country). Analyses in the observational studies were, if available, additionally adjusted for the statin dose by the natural logarithm of the dose equivalent as defined in Supplementary Table 3. This table shows the dose equivalent per statin type; dividing the statin dosage of an individual by the dose equivalent shown in Supplementary Table 3 will give the adjusted statin dosage.

Quality control and Meta-analysis

Centrally, within each study SNPs with minor allele frequency < 1% or imputation quality <0.3 were excluded from the analysis. QQ-plots were assessed for each study to identify between study differences (Supplementary Figure 1). The software package METAL was used for performing the meta-analysis [11]. A fixed effects, inverse variance weighted approach was used. Using an inverse variance weighted meta-analysis will give smaller weights to studies with large standard errors. To correct for possible population stratification, genomic control was performed by adjusting the within study findings and the meta-analysis results for the genomic inflation factor.

Second stage

SNPs with p-values $<5x10^{-4}$ in the first stage meta-analysis were selected for further investigation in a second stage. A maximum of two SNPs per locus were selected, based on statistical significance, except for the APOE locus, for which all genome-wide significant associated SNPs were selected for validation. A total of 246 SNPs,

within 158 independent loci, were selected for the second stage which was performed in the JUPITER trial, HPS study, and the Rotterdam Study, which all had GWAS data and response to statin treatment available. For two of the 246 SNPs a proxy was used in the JUPITER trial, and 31 SNPs were not available, nor was a proxy SNP. HPS provided data on 151 directly genotyped SNPs from GWAS and IPLEX experiments, including 48 of the requested SNPs and 103 proxy SNPs (r^2>0.8). Analyses in HPS were not adjusted for ln baseline LDL-C levels. In addition, the number of subjects with data varied from SNP-to-SNP and ranges from ~4000 for variants with GWAS data to ~18000 for some candidate genes. Results of the first and second stage were combined using fixed effects, inverse variance weighted meta-analysis and analyzed by METAL. As a third stage, six SNPs with p-values $5x10^{-8}<p<5x10^{-7}$ in the combined meta-analysis were selected for additional genotyping in the Scandinavian participants of the ASCOT study. Kaspar assays were designed for four of the SNPs using the KBioscience Primerpicker software, and oligos were provided by Intergrated DNA technologies (http://eu.idtdna.com/site). Full Kaspar methodology is available from LGC SNP genotyping (http://www.lgcgenomics.com/genotyping/kasp-genotyping-reagents/). Two SNPs (rs981844 and rs13166647) were genotyped using Taqman assays supplied by Life technologies (http://www.lifetechnologies.com/uk/en/home.html) using the standard Taqman protocol. Results of the additional genotyping were combined with results from the first and second stages using a fixed effects, inverse variance weighted meta-analysis and analyzed by METAL.

Determination of changes in LDL subfractions

LDL subclasses were analyzed as described previously [12] using non-denaturing gradient gel electrophoresis of fasting plasma samples taken at baseline and after 6 weeks of simvastatin 40 mg/d (CAP study, n=579) or 12 weeks of pravastatin 40 mg/d (PRINCE study, n=1284). Aliquots of 3.0 mL of whole plasma were mixed 1:1 with a sampling buffer of 20% sucrose and 0.25% bromophenol blue. Electrophoresis of samples and size calibration standards was performed using 2%–14% polyacrylamide gradients at 150 V for 3 hours following a 15-minute pre-run at 75 V. Gels were stained with 0.07% Sudan black for 1 hr and stored in a 0.81% acetic acid, 4% methanol solution until they were scanned by computer-assisted densitometry for determination of areas of LDL IVb (22.0–23.2 nm), LDL IVa (23.3–24.1 nm), LDL IIIb (24.2–24.6 nm), LDL IIIa (24.7–25.5 nm), LDL IIb (25.6–26.4 nm), LDL IIa (26.5–27.1 nm), and LDL I (27.2–28.5 nm). The cholesterol concentrations of the subfractions (mg/dL plasma) were determined by multiplying percent of the total stained LDL area for each subfraction by the LDL cholesterol for that sample [13]. For genetic association analyses, subfractions were grouped into large LDL (LDL I+IIa), medium

LDL (LDL IIb), small LDL (LDL IIIa) and very small LDL (LDL IIIb+IVa+IVb) as described previously [14]. A Generalized Estimating Equation method was used to test the association of log change with the interaction of the four SNPs by LDL subfraction.

Effect of off-treatment LDL-C

Effects of genetic variation on treatment response as measured by on-treatment LDL-C could be mediated through effects on the off-treatment LDL-C. To evaluate whether genetic on-treatment LDL-C likely reflects residual effect on off-treatment LDL-C, it is necessary to adjust for the off-treatment LDL-C levels and to correct the maximum likelihood estimate of the adjusted effect of genotype on on-treatment value for the noise in off-treatment values (the noise is both random measurement error and intra-individual variation in usual LDL-C). This analysis was only carried out in CARDS in which multiple baseline measurements were available. From the rules of path analysis, we calculated the direct effect γ of genotype on an on-treatment trait value as $\beta - \alpha\delta (1 - \rho) / \rho$, where β is the coefficient of regression for on-treatment trait value on genotype adjusted for measured off-treatment value, α is the coefficient of regression of baseline LDL on genotype, ρ is the intraclass correlation between replicate measurements of off-treatment values, and δ is the coefficient of regression for on-treatment value on observed off-treatment value [8]. For these calculations, we used $\rho = 0.8$ as a plausible value for the intra-class correlation based on the within-person correlation in LDL-C values taken over two off-treatment visits in CARDS.

The interaction of candidate SNPs with statin versus placebo allocation was assessed in the JUPITER trial, since this study was not involved in the first stage meta-analysis. Regression models were applied to the combined population of statin and placebo treated subjects by including extra terms encoding placebo allocation and the product of placebo allocation with SNP minor allele dose [7].

Genome-wide conditional analysis (GWCA) using Genome-Complex Trait Analysis (GCTA)

There may be multiple causal variants in a Gene and the total variation that could be explained at a locus may be underestimated if only the most significant SNP in the region is selected. To identify independent SNPS we ideally can perform a conditional analysis, starting with the top associated SNP, across the whole genome followed by a stepwise procedure of selecting additional SNPs, one by one, according to their conditional P values. Such a strategy would allow the discovery of more than two associated SNPs at a locus. To identify independent SNPs across the genome-wide data we used an approximate conditional and joint analysis approach implemented

in GCTA software [15]. We used summary level statistics from the first and second stage combined meta-analysis and LD corrections between SNPs estimated from CARDS GWAS data. SNPs on different chromosomes or more than 10Mb distant are assumed to be in linkage equilibrium. The model selection process in GCTA starts with the most significant SNP in the single-SNP meta-analysis across the whole genome with P value<5×10^{-7}. In the next step, it calculates the P-values of all the remaining SNPs conditional on the top SNP that have already been selected in the model. To avoid problems due to co-linearity, if the squared multiple correlations between a SNP to be tested and the selected SNP(s) is larger than a cut-off value, such as 0.9, the conditional P value for that SNP will be set to 1. Select the SNPs with minimum conditional P value that is lower than the cut-off P value. Fit all the selected SNPs jointly in a model and drop the SNPs with the P value that is greater than the cut-off P value. This process is repeated until no SNPs can be added or removed from the model.

Pathway analysis and construction of a Statin Response Network

Genes showing evidence of association (based on direct association or LD (HapMap CEU r^2>0.8)) were reviewed for evidence of involvement in statin response at a pathway level using GeneGo Metacore (Thomson Reuters (portal.genego.com)). A statin response network was constructed in two stages. Firstly all genes with a literature reported involvement in statin response (based on Medical subject headings (MeSH) were identified using GeneGo MetaCore (Supplementary Data 3). Secondly these genes were combined with all genes in associated loci (including genes in LD) and a network was constructed based on direct interactions only. By including direct interactions only, we created a conservative network of direct gene interactions that have been consistently linked to statin response in the literature.

Expression quantitative trait loci (eQTL) analysis

LDL-C associated index SNPs (246 SNPs) were used to identify 1443 LD proxy SNPs displaying complete linkage disequilibrium (r^2=1) across 4 HapMap builds in European ancestry samples (CEU) using the SNAP tool [16]. The primary index SNPs and LD proxies were searched against a collected database of expression SNP (eSNP) results including the following tissues: fresh lymphocytes [17], fresh leukocytes [18], leukocyte samples in individuals with Celiac disease [19], whole blood samples [20-23], lymphoblastoid cell lines (LCL) derived from asthmatic children [24;25], HapMap LCL from 3 populations [26], a separate study on HapMap CEU LCL[27], additional LCL population samples [28-30](Mangravite et al., unpublished), CD19+ B cells [31], primary PHA-stimulated T cells [28], CD4+ T cells [32], peripheral blood monocytes [31;33;34], CD11+ dendritic cells before and after Mycobacterium tuberculosis infection [35], omental and

subcutaneous adipose [20;30;36], stomach [36], endometrial carcinomas [37], ER+ and ER- breast cancer tumor cells [38], brain cortex [33;39;40], pre-frontal cortex [41;42], frontal cortex [43], temporal cortex [40;43], pons [43], cerebellum [40;43], three additional large studies of brain regions including prefrontal cortex, visual cortex and cerebellum, respectively (Emilsson, Cell in press), liver [36;44;45], osteoblasts [46], ileum [36;47], lung [48], skin [30;49] and primary fibroblasts [28]. Micro-RNA QTLs were also queried for LCL [50], and gluteal and abdominal adipose [51]. The collected eSNP results met the criteria for association with gene expression levels as defined in the original papers. In each case where a LDL-C associated SNP or proxy was associated with a transcript we further examined the strongest eSNP for that transcript within that dataset (best eSNP), and the LD between the best eSNP and GIST-selected eSNPs to estimate the concordance of the LDL-C and expression signals.

Statin response connectivity map analysis

The Connectivity Map (Cmap) data set is available at the Broad Institute (www.broadinstitute.org/cmap [52]) and contains more than 7000 expression profiles representing 1309 compounds used on five different cultured human cancer cell lines (MCF7, ssMCF7, HL60, PC3 and SKMEL5). We selected (prostate tumor-derived) PC3 cells as they showed the most responsiveness to statins at a genome wide level. Four statins were included in our analysis, including Pravastatin, Atorvastatin, Simvastatin and Rosuvastatin. PC3 Instance reference files for each statin treatment were extracted (as defined by Lamb et al.[52]), i.e. a treatment associated to its control pair. Transcripts were considered to show evidence of differential expression with a fold change >2. A fold change >1.5 was considered to be suggestive of differential expression only.

Exploration of functional impact among directly and indirectly associated variants

Genes and variants across all LDL-C associated loci were investigated for evidence of functional perturbation using a range of bioinformatics tools and databases. Variants showing LD (CEU r^2>0.8) with associated variants were explored for impact on coding gene function using Annovar [53] and regulatory function using a combination of HaploReg [54] and Regulomedb [55], which both draw on comprehensive data from the Encyclopedia of DNA Elements (ENCODE) [56] and the NIH Roadmap Epigenomics consortium [57]. Building on the functional annotation, we also identified variants which were shown to mediate eQTLs. Genes in associated loci were also used to query the NIH connectivity map for evidence of differential expression in PC3 cell lines treated with Pravastatin, Simvastatin and Rosuvastatin. By combining a wide range of functional data and pathway support we were able to build up a view of genes with the highest level of support in statin response.

Results

First stage meta-analysis

The GIST consortium includes six randomized controlled trials (n=8,421 statin recipients) and ten observational studies (n=10,175 statin recipients) that participated in the first stage (Methods, Supplementary Table 1 and 2 and Supplementary Note 1 and 2). To search for genetic variants associated with differential LDL-C response to statin therapy, each study independently performed a GWAS among statin users, using the difference between the natural log transformed LDL-C levels on- and off-treatment as the response variable (Methods).

The first stage meta-analysis identified three loci, including 13 SNPs, that attained genome-wide significance ($P<5x10^{-8}$) for association with LDL-C response to statin treatment (Figure 1 and Table 1). The most significant association was for a SNP on chromosome 19, at *APOE* (rs445925, MAF=0.098, β=-0.043, SE=0.005, $P=1.58 \times 10^{-18}$) (Figure 2A), indicating that carriers of the rs445925 SNP respond to statins with an additional 4.3% increase per allele in LDL-C lowering effect compared to non-carriers.

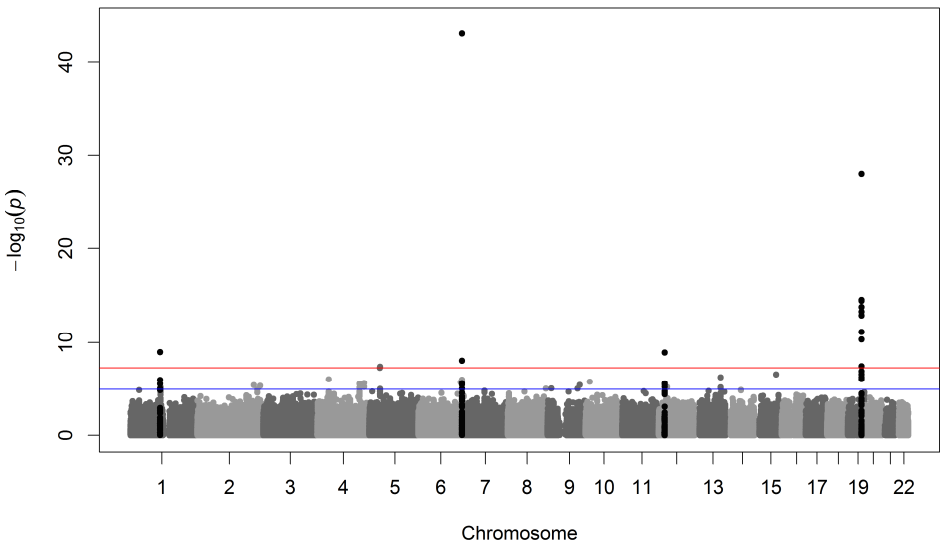

Figure 1. Results of the GWAS meta-analysis. Manhattan plot presenting the $-\log_{10}$ P-values from the combined meta-analysis (n=40914) on LDL cholesterol response after statin treatment. P-values were generated using linear regression analysis.

Table 1. Genome-wide significant associations in stage 1, stage 2, and combined meta-analysis

Chr	Position	Lead SNP	Gene	Coding allele	Non-coding allele	Phase	N	Frequency coding allele	Beta*	SE	% extra reduction#	P-value
1	109620053	rs646776	*SORT1/CELSR2/PSRC1*	C	T	Stage 1	16,697	0.230	-0.015	0.003	1.5	$6.70x10^{-7}$
						Stage 2	21,902	0.216	-0.010	0.003	1.0	$2.43x10^{-4}$
						Combined	38,599		-0.013	0.002	1.3	$1.05x10^{-9}$
6	160930108	rs10455872	*LPA*	G	A	Stage 1	12,981	0.069	0.041	0.006	-4.1	$1.95x10^{-11}$
						Stage 2	18,075	0.087	0.059	0.005	-5.9	$7.14x10^{-35}$
						Combined	31,056		0.052	0.004	-5.2	$7.41x10^{-44}$
12	21260064	rs2900478	*SLCO1B1*	A	T	Stage 1	16,749	0.165	0.016	0.003	-1.6	$2.26x10^{-6}$
						Stage 2	7,504	0.164	0.017	0.006	-1.7	$3.54x10^{-3}$
						Combined	24,253		0.016	0.003	-1.6	$1.22x10^{-9}$
19	50107480	rs445925	*APOE*	A	G	Stage 1	13,909	0.098	-0.043	0.005	4.3	$1.58x10^{-18}$
						Stage 2	3,613	0.157	-0.088	0.011	8.8	$1.41x10^{-15}$
						Combined	17,522		-0.051	0.005	5.1	$8.52x10^{-29}$

*Beta for difference between the natural log transformed on- and off-treatment LDL-C levels adjusted for natural log transformed off-treatment LDL-C, age, sex, and study specific covariates. The beta reflects the fraction of differential LDL-C lowering in carriers vs. non-carriers of the SNP; a negative beta indicates a better statin response (stronger LDL-C reduction), a positive beta a worse statin response. Betas and p-values were generated using linear regression analysis. #This percentage reflects the % extra LDL-C lowering in carriers vs. non-carriers of the SNP.

The second strongest association was with a SNP at *LPA* on chromosome 6 (rs10455872, MAF=0.069, β=0.041, SE=0.006, P=1.95 x 10^{-11}) (Figure 2B), indicating a 4.1% smaller LDL-C lowering per minor allele for carriers of the SNP compared to non-carriers. Associations at both loci have previously been described [7;8]. A third genome-wide significant association was found with a SNP at *RICTOR* on chromosome 5 (rs13166647, MAF=0.230, β=-0.253, SE=0.046, P=4.50 x 10^{-8}), although genotypes for this SNP were only available in two studies within the first stage (n=2,144).

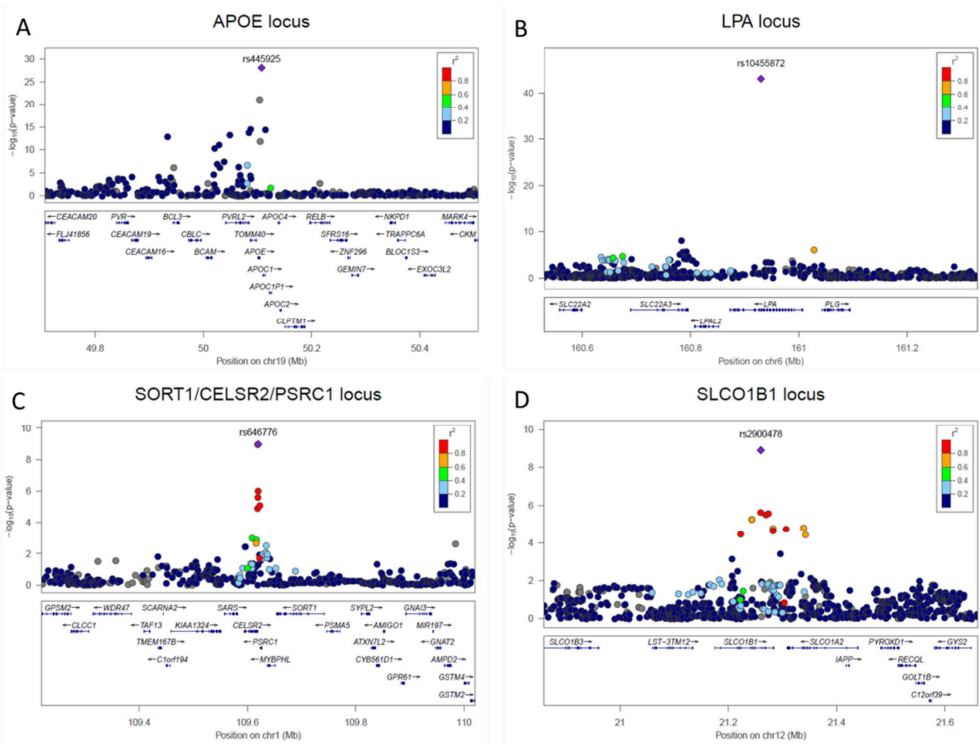

Figure 2. Regional association plots of the genome-wide significant associations with LDL cholesterol response after statin treatment. The plots show the genome-wide significant associated loci in the combined meta-analysis (n=40914), the *APOE* locus (A), the *LPA* locus (B), the *CELSR2* locus (C), and the *SLCO1B1* locus (D) (generated using LocusZoom [74]). The color of the SNPs is based on the LD with the lead SNP (shown in purple). The RefSeq genes in the region are shown in the lower panel. P-values were generated using linear regression analysis

Second stage meta-analysis

We selected 246 SNPs with P<5x10^{-4} from 158 loci for further investigation in three additional studies comprising up to 22,318 statin treated subjects (Methods, Supplementary Table 1 and 5 and Supplementary Note 3). This second stage

confirmed the genome-wide significant associations between variations within the *APOE* and *LPA* loci and LDL-C response, as being observed in the first stage (Table 1, Supplementary Figure 2 and Supplementary Table 5). In addition, SNPs at two new loci with p-values between $6.70x10^{-7}$ and $2.26x10^{-6}$ in the first phase, were shown to be significantly associated with statin induced LDL-C lowering after statin treatment in the total combined meta-analysis at a genome-wide level: *SORT1/CELSR2/PSRC1* (rs646776, β =-0.013, SE=0.002, $P=1.05x10^{-9}$) and rs12740374, β=-0.013, SE=0.002, $P=1.05x10^{-9}$) and *SLCO1B1* (rs2900478, β=0.016, SE=0.003, $P=1.22x10^{-9}$) (Figure 2C and 2D), indicating an additional 1.5% increase per allele in LDL-C lowering effect for carriers of the *SORT1/CELSR2/PSRC1* SNP and a 1.6% smaller LDL-C lowering per minor allele for carriers of the *SLCO1B1* SNP.

The six next-ranked SNPs with p-values just below $5x10^{-8}$ in the combined meta-analysis, including the two SNPs at *RICTOR* (rs13166647 and rs13172966) were selected for additional genotyping in the Scandinavian ASCOT participants (Methods). None of these six SNPs reached genome-wide significance after this additional genotyping (Supplementary Table 6). Therefore, our overall genome-wide significant findings were the SNPs at *APOE*, *LPA*, *SORT1/CELSR2/PSRC1*, and *SLCO1B1*.

Subfraction analyses

To extend our results for the novel GWAS finding *SORT1/CELSR2/PSRC1* we performed additional association analyses, using measurements of cholesterol levels in four LDL subfractions (large, medium, small, and very small) from two of the trials in GIST; CAP and PRINCE (Table 2 and Methods). The minor allele of *SORT1* rs646776 was associated with greater statin-induced reductions in levels of all LDL subfractions, and there was a non-significant trend for larger effect sizes and greater statistical significance for lowering of small and very small LDL (Table 2). In contrast, the *APOE* SNP associated with greater LDL-C response to statins (rs445925) showed a small and non-significant association with change in very small LDL (Table 2). For the minor allele of rs2900478 (*SLCO1B1*), the borderline significant association with smaller magnitude of LDL-C reduction showed a trend for preferential association with larger versus smaller LDL subfractions. The lack of association of rs10455872 (*LPA*) with changes in LDL subfractions is consistent with evidence discussed below that this locus affects levels of Lp(a) and not LDL particles. Using Generalized Estimating Equations, we tested the association of log change in each of the LDL subfractions with interactions of the four SNPs. For very small LDL, the association with the rs646776 minor allele was significantly different from that of the other minor alleles (P=0.03 after adjustment for multiple testing).

Table 2. Associations of the minor alleles of rs646776, rs445925, rs2900478, and rs10455872 with changes in LDL-C and LDL subfractions in response to statin in the combined CAP and PRINCE studies

	SORT1/CELSR2/PSRC1 rs646776 (MAF 0.2)			APOE rs445925 (MAF 0.086)			SLCO1B1 rs2900478 (MAF 0.16)			LPA rs10455872 (MAF 0.056)		
Change*	**Beta**	**SE**	**P-value**	**Beta**	**SE**	**P-value**	**Beta**	**SE**	**P-value**	**Beta**	**SE**	**P-value**
LDL-C total	-0.023	0.008	0.003	-0.046	0.018	0.008	0.010	0.005	0.04	0.032	0.019	0.09
Large LDL-C	-0.028	0.014	0.042	-0.075	0.029	0.009	0.02	0.008	0.01	0.036	0.031	0.23
Medium LDL-C	-0.027	0.015	0.075	-0.079	0.032	0.012	0.016	0.009	0.07	0.010	0.034	0.77
Small LDL-C	-0.047	0.018	0.009	-0.071	0.037	0.050	0.002	0.010	0.83	-0.024	0.039	0.54
Very small LDL-C	-0.034	0.009	0.00006	-0.022	0.017	0.202	0.001	0.005	0.90	0.008	0.019	0.67

*Change: ln (on treatment) - ln (baseline). Models adjusted for log (baseline variable), age, sex, BMI, smoking(y/n), study (CAP vs. PRINCE). Betas and p-values were assessed using a Generalized Estimating Equation method.

Effects of off-treatment LDL-C

To demonstrate that our findings for LDL-C response to statin treatment are unlikely to be explained through associations with baseline LDL-C levels, we performed a number of additional analyses (Methods). First, Supplementary Table 7 shows regression coefficients for baseline-adjusted and measurement noise-corrected estimates of the direct effect of genotype on on-treatment LDL-C at the strongest SNPs in the GIST meta-analysis ($P<1 \times 10^{-8}$) which were available in the CARDS dataset. Correcting our effect size estimate further and modeling measurement noise at baseline reduced the apparent effect only slightly for all the markers, suggesting that there is little effect of measurement noise. Next, within the JUPITER trial, additional analyses were performed to determine whether there was an interaction between LDL-C change and statin or placebo allocation. Supplementary Table 8 shows significant P-values for interaction (all $<5 \times 10^{-2}$) for SNPs at the four genome-wide significant loci in the GIST meta-analysis, also suggesting that genetic effects on baseline LDL-C as manifest in the placebo group contribute at most only in part to genetic effects on LDL-C response in the statin group.

Genome-Wide Conditional Analysis

To investigate if there were multiple SNPs within any gene and multiple loci associated with differential LDL-C lowering to statin therapy we performed a conditional analysis across the genome using the summary statistics of the combined meta-analysis. The results of the Genome-Wide Conditional Analysis (GWCA) (Methods, Supplementary Table 9) showed 14 SNPs independently associated with statin response and these explained approximately 5% of the variation in LDL-C response to statin treatment. Of the 14 independent SNPs, six were genome-wide significant in the combined GWAS meta-analysis (Supplementary Table 5).

Previous findings

In Supplementary Table 10 we performed a look-up in our GWAS meta-analysis for SNPs previously described in the literature (NHGRI Catalogue [58] of Published GWAS and Candidate gene studies) to be associated with statin response, besides the loci associated at a genome-wide level in the current study. None of these SNPs was associated with statin response in our GWAS after correcting for multiple testing.

Functional analyses

Functional characterization of the 246 SNPs selected for the second stage was performed using a range of bioinformatics tools (Methods). A total of 420 eQTL associations were identified across a wide range of tissues (Supplementary Data 1), which comprised 67 independent gene eQTL associations. Eleven genes, including

APOE, SORT1, CELSR2 and *PSRC1* showed eQTLs in liver, which considering its primary role in mediating statin-induced LDL reduction, may be particularly relevant to statin response. Putative gene eQTLs were combined with genes annotated to variants in LD with LDL-C response associated variants, resulting in a list of 185 candidate gene loci, defined by 2681 SNPs (Supplementary Data 2 and 3). In order to identify statin responsive genes among the candidate loci, gene expression data measured in response to statin treatment in a range of cell lines was retrieved from the Connectivity Map resource [52] (Methods). Five genes (*APOE, BRCA1, GRPEL1, ADRB2* and *ETV1*) showed convincing evidence of statin responsiveness on the basis of >2-fold differential expression in response to statin treatment. Eight genes showed suggestive evidence (1.5-2-fold change; *TOMM40, SREBP1, PSRC1, BCL3, BCAM, ANK3, SIVA1*, and *RANBP9*) (Supplementary Data 3).

Finally, involvement in statin response was investigated at a pathway level using GeneGo Metacore (Thomson Reuters [59]). Briefly, 87 literature reported genes linked to statin response were combined with the 185 candidate gene loci reported here (Supplementary Data 3). A conservative network of direct interactions was constructed between query genes (Supplementary Data 4). The network included 24 genes located in the LDL-C associated loci (Supplementary Figure 4). Collectively our functional and pathway analysis confirms a strong biological and functional role in statin response for several strongly associated gene loci, including *APOE/TOMM40/PVRL2*, and *SORT1/CELSR2/PSRC2.*

Discussion

We have performed a meta-analysis of GWAS including more than 40,000 subjects, investigating genetic variants associated with variation in LDL-C lowering upon statin treatment independent from associations with baseline LDL-C. We identified four loci at genome-wide significance, including the previously identified *APOE* and *LPA,* and the novel GWAS loci *SORT1/CELSR2/PSRC1,* and *SLCO1B1*.

Nine SNPs in the *APOE* gene region reached genome-wide significance for LDL-C response. The minor allele of the lead SNP rs445925, which is a proxy for the apoE ε2 protein variant defining SNP rs7412 [60], was associated with a larger LDL-C lowering response to statins compared to carriers of the major allele. The magnitude and direction of the effect size was similar to previously reported findings for the rs445925 variant in the GWAS study performed in CARDS and ASCOT [8] and of the SNP rs7412 in JUPITER [7]. Since the apoE ε2 protein results in increased hepatic

cholesterol synthesis, it may also predispose to stronger inhibition of cholesterol synthesis by statin treatment [8;10].

Three independent SNPs at *LPA* were significantly associated with LDL-C response to statins. The minor G allele of the lead SNP rs10455872 was associated with smaller LDL-C reduction than the major allele. This result was similar to the previous GWAS findings for this SNP in the JUPITER trial and the combined ASCOT and CARDS study [7;8]. The rs10455872 SNP was strongly associated with the KIV-2 copy number variant in lipoprotein(a) (Lp(a)), which encodes variability in apo(a) size and is responsible for approximately 30% of variance in Lp(a) levels [8;61]. Furthermore, rs10455872 was shown to be strongly associated with plasma Lp(a) levels [62]. Standard assays of LDL-C, as well as the Friedewald formula, include cholesterol that resides in Lp(a) [6;8]. Carriers of this *LPA* variant are characterized by higher Lp(a) levels and a larger proportion of their measured LDL-C resides in Lp(a) particles [8;10]. Since statin therapy does not reduce the number of Lp(a) particles [63], their presence attenuates the measured LDL-C response to statins.

Two SNPs at *SORT1/CELSR2/PSRC1* (rs646776 and rs12740374) on chromosome 1p were associated with an enhanced statin LDL-C response. A similar association was previously observed in a large candidate-gene study in HPS [6], however, we demonstrate this finding now first at a genome-wide significance level. The minor allele of rs12740374 has been shown to generate a binding site for the transcription factor C/EBPa [14]. Transcription results in up-regulation of hepatic expression of three genes at this locus, *SORT1, CELSR2* and *PSRC1* [14], which we also showed in our eQTL analysis (Supplementary Data 1). Of these, *SORT1* is most notable, in that it encodes the multifunctional intracellular trafficking protein sortilin, which has been shown to bind tightly to apoB [64]. Sortilin-induced lowering of plasma LDL-C results from two mechanisms: reduced secretion of apoB-containing precursors, and, perhaps of greater importance, increased hepatic LDL uptake via binding to sortilin at the cell surface, with subsequent internalization and lysosomal degradation [64]. Notably, the minor allele of rs646776 is preferentially associated with lower levels of small and very small LDL (Table 2), suggesting that sortilin is of particular importance for regulating levels of these particles [14]. Smaller LDL subfractions have been shown to be relatively enriched in particles with reduced LDL receptor binding affinity and cellular uptake [65], a property that may contribute to their associations with increased risk for cardiovascular disease [13]. This property may also underlie the diminished efficacy of statins for reduction of these particles (Supplementary Figure 3) [66], since statins act to reduce LDL-C levels to a large extent by increasing LDL receptor expression as a result of upregulation of the transcription factor SREBP2, whereas

SORT1 is not regulated by this mechanism. Hence, the greater statin-mediated reduction of LDL-C among carriers of the rs646776 minor allele could be attributed to relative depletion of LDL particles dependent on sortilin for clearance and hence a residually greater proportion of those LDL particles whose uptake is more dependent on the LDL receptor than on sortilin.

Notably, the strong association of rs646776 with statin-induced reductions in small and very small LDL particles contrasts to the weaker associations of changes in these particles with rs445925, likely the result of differing mechanisms underlying the effects of these SNPs on statin response. As noted above, rs445925 is a proxy for the SNP defining the apoE ε2 protein variant that is thought to predispose to heightened statin response as a result of greater statin inhibition of cholesterol synthesis and hence upregulation of SREBP and LDL receptor activity.

The *SLCO1B1* rs2900478 minor allele was associated with a smaller LDL-C reduction in response to statin treatment. *SLCO1B1* encodes the organic anion-transporting polypeptide OATP1B1 and facilitates the hepatic uptake of statins [67]. SNP rs2900478 is in strong LD (r^2=0.89) with rs4149056, which represents the Val174Ala substitution resulting in complete loss of function. In the HPS trial, which used simvastatin, this candidate-gene SNP was associated with a 1% lower LDL-C reduction per allele [6]. Single-dose studies have shown that the observed area under the curve of plasma level of active simvastatin after a dose of 40mg was 221% higher in rs4149056 CC homozygotes compared with rs4149056 TT homozygotes, as compared to atorvastatin 20 mg (144% higher for CC vs TT) and rosuvastatin 40mg (117% higher for CC vs TT) [68]. This finding results from the slower hepatic uptake of statins caused by the genetic variant, which would also be expected to result in a reduction of the cholesterol lowering effect [69]. In a GWAS of the genetic risk factors for simvastatin-induced myopathy, *SLCO1B1* showed the strongest association [69]. Homozygous carriers of the *SLCO1B1* variant had a 16.9-times higher risk for myopathy compared to non-carriers. This might have led to a decrease in study medication adherence, and consequently a decreased effect on LDL-C in carriers of this SNP. In addition, previous analysis in the GoDARTS study showed that the effect of the SLCO1B1 gene on statin efficacy was abolished after removal of individuals who showed signs of intolerance [70].

GWCA identified three independent loci in the *APOE* gene region and two loci in the *LPA* gene region (Supplementary Table 9). GWCA also showed several other loci with $P<5x10^{-8}$, that were not GWAS significant on single SNP analysis (*HGD, RNF175, ISCA1L-HTR1A, GLIS3-SLC1A1, LOC100128657, NKX2-3-SLC25A28*, and *PELI2*). These

findings will require replication in independent, larger datasets. The significant SNPs in the GWCA analysis explained approximately 5% of the variation in LDL-C response to statin treatment. Whether this 5% is clinically relevant should be investigated by other studies. For example it would be of interest to investigate if this differential LDL-C lowering is also associated with differential event reduction by statin treatment.

In the current study we combined the results of six randomized clinical trials and ten observational studies in the first stage. This approach resulted also in combining several types of statins, since different statins were studied in the trials and within the observational studies (Supplementary Table 2). This, and the variation in statin dosage during follow-up for an individual, is a limitation of the current study, since for example the impact of the *SLCO1B1* variant on statin pharmacogenetics is known to be highly dependent on statin type and dose [68;71]. To overcome this limitation, the individual study analyses were adjusted for statin dose. Dividing the actual statin dose given by the statin specific dose equivalent (Supplementary Table 3) gives the statin adjusted equivalent based on the daily dosages required to achieve a mean 30% LDL-C reduction. Using this table we made the different statin dosages and types comparable within the studies. To correct for between study variance we used a fixed effects meta-analysis with inverse variance weighting. Since we observed that the *SLCO1B1* gene was genome-wide significantly associated with LDL lowering, this highlights the thoroughness of our analytical approach, in which the analyses were correctly adjusted for the type and dose of statins used (Supplementary Table 3). Moreover, a comparison of the estimates of the SNPs between the RCTs (where there are no intra-individual differences in dosages) with the estimates of the SNPs in the observational studies showed large homogeneity between the estimates in the various study designs (Supplementary Figure 2), indicating that our adjustment for dosage seems to be sufficient within this study.

Another possible limitation of the current study is the influence of the identified genetic variants on baseline LDL-C levels. In pharmacogenetic studies investigating the LDL-C lowering response to statins it is important to eliminate the effect of association between the genetic variant and baseline LDL-C levels, since those findings may confound the response to treatment associations. Previous large GWAS studies have shown strong associations between baseline LDL-C levels and genetic variants in *SORT1/CELSR2/PSRC1*, *APOE*, and *LPA* [72]. To eliminate those possible confounding effects our response to treatment analyses were adjusted for baseline LDL-C levels. In addition, additional analysis in CARDS and JUPITER suggests no or

little influence of genetic effects on baseline LDL-C on the genetic effects on LDL-C lowering response.

In conclusion, this study is the largest meta-analysis of GWAS for LDL-C response to statin therapy conducted to date. Our results demonstrate that apart from the previously identified *APOE* and *LPA* loci, two new loci, *SORT1/CELSR2/PSRC1* and *SLCO1B1*, also have a modest but genome-wide significant effect on LDL-C response. The minor alleles of the *APOE* rs445925 and *SORT1/CELSR2/PSRC1* rs646776 SNPs were associated with a larger statin response, whereas the minor alleles of the *LPA* rs10455872 and *SLCO1B1* rs2900478 SNPs, were associated with a smaller statin response. Our findings advance the understanding of the pharmacogenetic architecture of statin response.

Supplementary material

Supplementary material is available at *Nature Communications* online.

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AGES
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Framingham HS

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engaged in the discovery, development, manufacture and marketing of proprietary therapeutics such as rosuvastatin, but do not consider that this creates any conflict of interest with the subject-matter of this publication. RMK serves on the Merck Global Atherosclerosis Advisory Board. The remaining authors declare no competing financial interests.

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CHAPTER 5

In search for genetic determinants of clinically meaningful differential cardiovascular event reduction by pravastatin in the PHArmacogenetic study of Statins in the Elderly at risk (PHASE)/PROSPER study

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Abstract

Statin therapy is widely used in the prevention and treatment of cardiovascular events and is associated with significant risk reductions. However, there is considerable variation in response to statin therapy both in terms of LDL cholesterol reduction and clinical outcomes. It has been hypothesized that genetic variation contributes importantly to this individual drug response. In this study, we investigated the interaction between genetic variants and pravastatin or placebo therapy on the incidence of cardiovascular events by performing a genome-wide association study in the participants of the PROspective Study of Pravastatin in the Elderly at Risk for vascular disease – PHArmacogenetic study of Statins in the Elderly at risk (PROSPER/PHASE) study (n=5244). We did not observe genome-wide significant associations with a clinically meaningful differential cardiovascular event reduction by pravastatin therapy. In addition, SNPs with p-values lower than 1×10^{-4} were assessed for replication in a case-only analysis within two randomized placebo controlled pravastatin trials, CARE (n=711) and WOSCOPS (n=522). rs7102569, on chromosome 11 near the *ODZ4* gene, was replicated in the CARE study (p=0.008), however the direction of effect was opposite. This SNP was not associated in WOSCOPS. In addition, none of the SNPs replicated significantly after correcting for multiple testing. We could not identify genetic variation that was significantly associated at genome-wide level with a clinically meaningful differential event reduction by pravastatin treatment in a large prospective study. We therefore assume that in daily practice the use of genetic characteristics to personalize pravastatin treatment to improve prevention of cardiovascular disease will be limited.

Introduction

Cardiovascular disease is the leading cause of death in industrialized countries [1]. Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), are the most prescribed class of drug worldwide and are widely used in the prevention and treatment of cardiovascular disease. Statin therapy is in general associated with a low-density lipoprotein (LDL)-cholesterol lowering of 30-55% [2] and a reduction of cardiovascular events of 20-35% [3]. However, there is considerable variation in response to statin therapy both in terms of LDL cholesterol lowering and clinical outcomes.

Recently, results from six genome-wide association studies (GWAS) on the pharmacogenetics of statin therapy have been published [4], mainly focusing on the lipid lowering effects after statin therapy [5-10]. Evidence from the studies focusing on the lipid lowering response suggests that genetic variations in the apolipoprotein E (*APOE*) and apolipoprotein(a) (*LPA*) genes are associated with differential LDL cholesterol lowering after statin treatment [5-8;10]. The GWAS in 85 myopathy subjects and 90 controls treated with simvastatin identified the *SLCO1B1* gene variants to be associated with statin-induced myopathy [9]. However, none of these GWAS studies reported the pharmacogenetic effects of statins on cardiovascular events.

The association between genetic variants and the incidence of cardiovascular events after statin therapy has been investigated mainly by candidate gene studies. For example, several studies have reported an association between genetic variants in *KIF6* and event reduction after statin treatment [11-13]. However, these results are equivocal and could not be replicated in other studies [14;15]. Within the WOSCOPS and CARE trials, a case-only GWAS was performed to identify genetic loci associated with differential cardiovascular event reduction by pravastatin therapy. Genetic variation within the DNAJC5B gene was significantly associated and replicated in the PROSPER study [16]. To identify genetic variants associated with a clinically meaningful differential cardiovascular event reduction by pravastatin or placebo treatment on a genome-wide level, we conducted a GWAS in the PHArmacogenetic study of Statins in the Elderly at risk (PHASE) [17]. The PHASE project is a GWAS conducted in the PROspective Study of Pravastatin in the Elderly at Risk for vascular disease (PROSPER) [18]. The results were assessed for replication in two independent prospective pravastatin 40 mg trials, the Cholesterol and Recurrent Events (CARE) trial [19] and the West of Scotland Coronary Prevention Study (WOSCOPS) [20].

Methods

Study population

PROSPER was an investigator-driven, prospective multi-national randomized placebo-controlled trial to assess whether treatment with pravastatin diminishes the risk of major vascular events in the elderly [18;21]. Between December 1997 and May 1999, subjects in Scotland (Glasgow), Ireland (Cork) and the Netherlands (Leiden) were screened and enrolled. Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or were at increased risk for such disease because of smoking, diabetes, or hypertension. A total number of 5804 subjects were randomly assigned to pravastatin or placebo. The primary endpoint in PROSPER was the combined endpoint of death from coronary heart disease (CHD), non-fatal myocardial infarction (MI), and occurrence of clinical stroke, either fatal or non-fatal. All endpoints were adjudicated by the study Endpoint Committee. The protocol of the PROSPER study was approved by the medical ethics committees of each participating institution. Written informed consent was obtained from all participating subjects.

The PHASE project is a genome-wide association study (GWAS) in the participants of the PROSPER study, to investigate the genetic variation responsible for the individual variation in drug response, and has been described previously [17]. The study was sponsored by the European Union's Seventh Framework Programme (FP7/2007-2013), under grant agreement number HEALTH-F2-2009-223004.

Genotyping

The genotyping and quality control performed in the PHASE project has been described previously [17]. In brief, genotyping was conducted using the Illumina 660-Quad beadchips following manufacturer's instructions. After a stringent quality control (call rate< 95%) 557,192 SNPs in 5244 participants were available for analysis [17]. Those SNPs were imputed up to 2.5 million autosomal CEPH HapMap SNPs using MaCH imputation software based on the HapMap built 36 release 22.

Statistical Analysis

Genome wide association analysis was performed with ProbABEL software specialized in genetic association analysis with imputed data taking the probability of the genotype into account [22]. For the current analysis we assessed the interaction between genetic variants and statin treatment (pravastatin or placebo) on the incidence of the primary endpoint using a logistic regression model. To estimate the differential event reduction by pravastatin an interaction term between treatment

(pravastatin or placebo) and SNP was included in the model. By including this interaction term in the logistic regression model we can estimate the difference in statin treatment effect in carriers and non-carriers of a SNP. The model was adjusted for sex and age, and country to correct for the within-study population structure. To reduce the probability of false positive findings by multiple testing, a Bonferroni correction was used. The p-value threshold for genome-wide significant results was set at 5.0×10^{-8}.

Power calculation

The main aim of the current study was to identify genetic variants associated with clinically meaningful differential event reduction after pravastatin therapy. To detect genetic variants relevant for clinical practice we used a relative large minor allele frequency (MAF), large genetic effect, and large interaction effect for the power calculation. Power calculations for detecting clinically meaningful interactions between genetic variants and pravastatin treatment were performed using Quanto software (http://hydra.usc.edu/gxe). Based on a total number of 800 cases with the primary endpoint, we calculated that with a MAF of 25% in a log additive model, a prevalence of 50% of the environmental factor (pravastatin use) and a baseline risk of the primary event of 10%, a pravastatin effect of 0.8, a gene effect of 1.5, and an interaction effect of 2.0, the statistical power to detect the interaction between gene and environment is 80% for a p-value threshold of 5×10^{-7}.

The power to detect smaller, but from the biological point of view perhaps also interesting effects (interaction effect of 1.2) was limited (<10%), and therefore this was not the purpose of the current investigation.

Replication

From each independent locus associated with p-values $<1.0 \times 10^{-4}$ for interaction from our logistic regression analysis, two SNPs were assessed for replication in two independent cohorts, the CARE trial [19] and the WOSCOPS study [20]. The CARE trial was a double blind randomized placebo-controlled clinical trial in which 4159 post-myocardial infarction patients (age range 21-75 years) were treated with 40 mg pravastatin (N=2081) or placebo (N=2078). The WOSCOPS study was a double blind randomized placebo-controlled clinical trial in which 6595 men (age range 45-64 years) with hypercholesterolemia and no history of myocardial infarction were treated with 40 mg pravastatin (N=3302) or placebo (N=3293). For the current replication, we used data of the GWAS performed in all who had a cardiovascular disease event (a composite endpoint of death from CHD, non-fatal MI, revascularization procedures, or stroke) of the CARE (n=711) and WOSCOPS (n=522)

trials. In both CARE and WOSCOPS a case-only analysis was used to calculate the p-value of the Synergy Index [23]. The Synergy Index is an estimate for the interaction between pravastatin therapy and genotype that would be observed in a study that included both cases and non-cases. For 64 SNPs replication was requested, data was available for 47 SNPs, 22 with identical rs-number as the SNPs in PROSPER/PHASE and 25 SNPs in LD (r^2>0.8). Of the remaining 17 SNPs, 10 SNPs were genotyped in CARE using PCR assays (9 identical to PROSPER/PHASE SNPs, 1 SNP in LD). Of these 10 SNPs, one SNP was associated with an interaction p-value < 0.1 and was also genotyped in the entire WOSCOPS cohort. Seven of the 64 SNPs were not genotyped in either CARE or WOSCOPS.

Results

Table 1 shows the baseline characteristics of the 5244 subjects participating in the PROSPER/PHASE study, stratified by the allocation to pravastatin or placebo. Subjects allocated to pravastatin were similar compared to placebo treated subjects. The mean age of all subjects at study entry was 75.3 years and about 50% of the

Table 1. Baseline characteristics of the PROSPER/PHASE study stratified by pravastatin treatment

	Placebo (n=2639)	Pravastatin (n=2605)
Continuous variables (mean, SD)		
Age (years)	75.3 (3.3)	75.4 (3.4)
Systolic blood pressure (mmHg)	154.6 (21.9)	154.5 (21.9)
Diastolic blood pressure (mmHg)	84.0 (11.7)	83.4 (11.1)
Body mass index (kg/m^2)	26.8 (4.3)	26.8 (4.1)
Total cholesterol (mmol/L)	5.7 (0.9)	5.7 (0.9)
LDL cholesterol (mmol/L)	3.8 (0.8)	3.8 (0.8)
HDL cholesterol (mmol/L)	1.3 (0.3)	1.3 (0.4)
Triglycerides (mmol/L)	1.5 (0.7)	1.6 (0.7)
Categorical variables (n, %)		
Males	1267 (48.0)	1257 (48.3)
Current smoker	720 (27.3)	672 (25.8)
History of hypertension	1630 (61.8)	1627 (62.5)
History of diabetes	279 (10.6)	265 (10.2)
History of angina	682 (25.8)	742 (28.5)
History of claudication	173 (6.6)	181 (6.9)
History of myocardial infarction	361 (13.7)	347 (13.3)
History of stroke or TIA	291 (11.0)	295 (11.3)
History of vascular disease*	1145 (43.4)	1191 (45.7)

* Any of stable angina, intermittent claudication, stroke, transient ischemic attack (TIA), myocardial infarction, peripheral artery disease surgery, or amputation for vascular disease more than 6 months before study entry.

participants were female. For the participants with GWAS data, the mean duration of follow-up was 3.2 years and the number of primary events was 798, 434 events in the placebo group and 364 events in the pravastatin group. Within the PHASE project, pravastatin reduced the incidence of primary events by 17% (age, sex, and country adjusted hazard ratio 0.83 [95% CI: 0.72-0.96]), which is comparable to the previous reported hazard ratio in the total PROSPER population (HR 0.85 [95% CI: 0.74-0.97]) [18].

In figure 1 the QQ-plot of the interaction p-values is shown (lambda = 0.996). The QQ plot and lambda do not indicate an excess of significant results compared with those expected by chance, and indicate sufficient control for possible population stratification.

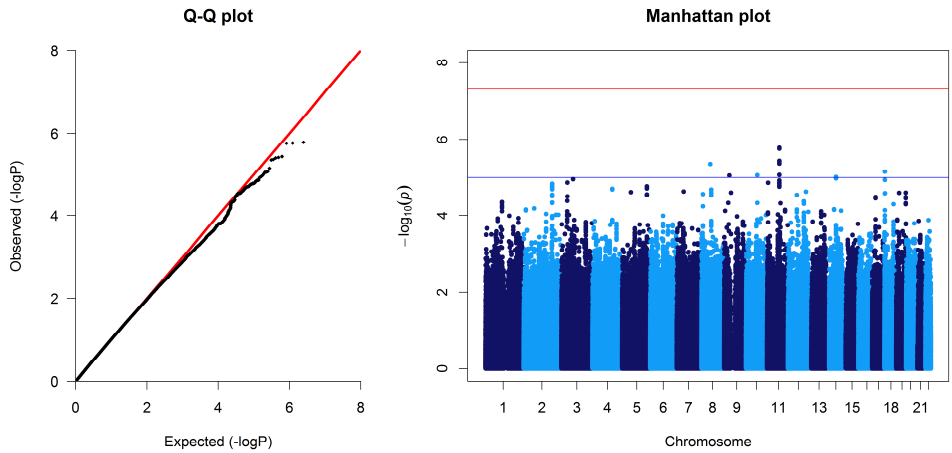

Figure 1. Q-Q and Manhattan plot for GWAS on the interaction between genotype and pravastatin treatment on the primary endpoint in the PROSPER/PHASE study. The left panel shows the quantile-quantile (QQ) plot of the observed versus expected p-values. The right panel shows the Manhattan plot, presenting the –log10 p-values of the interaction p-values.

Figure 1 shows the results of the GWAS assessing the interaction between genetic variants and statin treatment on the incidence of the primary endpoint depicted in a Manhattan plot. We did not observe genome wide significant associations, indicating that all estimates for the cardiovascular event reduction by pravastatin treatment were similar between SNP carriers and non-carriers. Furthermore, none of the SNPS reached the p-value of 5×10^{-7}, for which the power (as indicated) would have been 80%. Loci with low p-values (p-value $<1 \times 10^{-5}$) were found around *ADAMTS14* (chr 10) and *PPP2R5E* (chr 14), and near *ODZ4* (chr 11), *XKR4* (chr 8), *METTL4* (chr 18),

Table 2. Genomic loci interacting with statin treatment with a p-value smaller than 1E-04

CHR	Position	Top SNP	A1	A2	Frq A1	Beta	SE	p-value	Gene or nearby genes
1	107319828	rs12725107	A	C	0.80	0.57	0.14	4.50E-05	LOC126987, PRMT6
2	13334109	rs453359	C	A	0.57	-0.57	0.14	7.05E-05	TRIB2, FAM84A
2	67604877	rs17633730	G	T	0.96	1.49	0.37	6.59E-05	ETAA1, LOC402076
2	143476854	rs352887	A	G	0.47	-0.43	0.11	9.18E-05	KYNU
2	182620241	rs1913896	T	C	0.76	-0.58	0.13	1.47E-05	PPP1R1C
2	206775723	rs17223074	C	T	0.96	-1.42	0.36	9.60E-05	GPR1
2	222230114	rs1519483	C	T	0.72	0.55	0.14	9.67E-05	LOC402120, LOC646444
3	37487651	rs11709385	C	G	0.65	-0.50	0.12	1.37E-05	ITGA9
3	48706491	rs3172494	G	T	0.87	-0.64	0.16	9.70E-05	IHPK2
3	74427932	rs502114	A	T	0.53	-0.48	0.11	1.12E-05	CNTN3
4	126962141	rs12501068	G	T	0.96	-1.48	0.35	1.98E-05	LOC645841, LOC132817
5	55445178	rs160919	A	G	0.27	-0.52	0.12	2.43E-05	ANKRD55
5	158689546	rs6894567	A	G	0.80	-0.61	0.14	1.71E-05	IL12B
7	43414315	rs10255565	T	C	0.82	-0.78	0.18	2.35E-05	HECQ1
8	9374196	rs6601319	T	C	0.26	0.57	0.14	2.73E-05	LOC100129150, TNKS
8	56077010	rs3934874	G	A	0.81	-0.68	0.15	4.43E-06	LOC100128419, XKR4
8	62975272	rs1116816	C	T	0.69	0.52	0.12	2.10E-05	ASP, LOC645551
9	5520017	rs7870226	G	A	0.38	0.45	0.11	7.63E-05	PDCD1LG2
9	25629941	rs6475859	A	C	0.79	0.59	0.14	4.70E-05	C9orf134, TUSC1
9	32878825	rs10971182	G	A	0.92	1.18	0.27	8.87E-06	TMEM215, ASSP12
9	43582384	rs10907653	T	C	0.71	-2.68	0.64	2.52E-05	FAM75A6, CNTNAP3B
10	72186144	rs2791188	A	G	0.82	0.67	0.15	8.76E-06	ADAMTS14
10	127991998	rs868589	T	C	0.75	0.48	0.12	9.71E-05	ADAM12
11	6384682	rs1079199	T	C	0.69	0.52	0.12	1.38E-05	APBB1
11	69857496	rs655130	C	T	0.94	-1.32	0.34	9.43E-05	PPFIA1
11	78862547	rs7102569	G	A	0.35	0.55	0.12	1.65E-06	ODZ4, LOC646112
11	86414173	rs7927570	G	T	0.84	-0.64	0.16	4.80E-05	FZD4, TMEM135
11	116182933	rs1263167	A	G	0.81	0.56	0.14	7.16E-05	APOA5, APOA4
12	56811397	rs17120361	G	A	0.96	-1.45	0.35	2.88E-05	XRCC6BP1, LOC100127973
12	102208363	rs10778228	G	A	0.66	0.48	0.12	4.25E-05	LOC728545, C12orf42
12	115701524	rs4767452	C	T	0.91	-0.88	0.21	2.37E-05	RNFT2
12	130358787	rs7135770	C	T	0.74	0.79	0.20	8.06E-05	LOC100128002, LOC338797
13	80897164	rs9545683	C	T	0.90	-0.75	0.19	7.86E-05	LOC100129023, PTMAP5
13	101074718	rs1436260	A	G	0.28	-0.49	0.13	9.76E-05	ITGBL1
14	49550710	rs11157718	T	C	0.25	0.50	0.13	6.92E-05	LOC283551, PDLIM1P

Table 2. Genomic loci interacting with statin treatment with a p-value smaller than 1E-04 (*continued*)

CHR	Position	Top SNP	A1	A2	Frq A1	Beta	SE	p-value	Gene or nearby genes
14	63044403	rs1271562	A	T	0.88	0.77	0.17	9.68E-06	PPP2R5E
14	95377650	rs1885155	C	A	0.23	0.52	0.13	7.55E-05	LOC100133207, LOC730125
17	21932776	rs11654492	A	C	0.49	-0.46	0.11	3.28E-05	LOC100131001
18	2384986	rs7242734	G	T	0.88	-0.84	0.19	7.05E-06	LOC100128360, METTL4
18	4403363	rs281018	C	T	0.94	-2.12	0.50	2.58E-05	LOC284215, PPIAP14
18	58924818	rs8092360	C	T	0.81	0.58	0.14	5.12E-05	PHLPP, BCL2
19	18203477	rs271828	C	A	0.62	-0.49	0.12	2.55E-05	PDE4C
19	61187205	rs306468	A	T	0.55	0.47	0.11	2.51E-05	NLRP8

Abbreviations: A1, coding allele; A2, non-coding allele; CHR, Chromosome; SNP, Single Nucleotide Polymorphism; Frq coding allele, Frequency non-reference allele; SE, standard error.

and *TMEM215* (chr 9) (Table 2). Overall we observed 43 loci, including 140 SNPs, possibly associated with differential event reduction after pravastatin or placebo treatment (p-values $<1 \times 10^{-4}$). None of those loci were previously reported to be associated with cardiovascular diseases. Stratifying the analysis for participants with and without a history of vascular disease before participating in the PROSPER study did not change the results (data not shown).

In supplementary table 1 we show all loci with p-values for the interaction term (SNP x drug) $<1x10^{-4}$ and the corresponding p-values for the intrinsic SNP effect. None of the SNPs with a p-value for interaction term $< 1x10^{-4}$ reached genome-wide significance for the intrinsic SNP effect. In supplementary table 2 we show all loci with p-values for the intrinsic SNP effect $<1x10^{-4}$ and the corresponding p0values for the interaction term (SNP x drug). No SNPs reached genome-wide significance for the intrinsic SNP effect.

Loci associated with p-values $<1 \times 10^{-4}$ were assessed for replication in the CARE trial and the WOSCOPS study. The results of the replication are shown in table 3. Only rs7102569 was significantly associated in the CARE trial (p=0.008). Rs7102569 is present on chromosome 11 and close to the *ODZ4* gene and *LOC646112* pseudo-gene. In PROSPER/PHASE rs7102569 was associated with $p=1.65 \times 10^{-6}$, in CARE a proxy for this SNP, rs11237851, was used and associated with a p-value of 0.008. However, the direction of the effects was opposite in CARE compared to PROSPER and after correcting for multiple testing the association did not remain significant.

Table 3. Replication of most significant loci (p<1E-04) in CARE trial and WOSCOPS study

CHR	TopSNP PROSPER/ PHASE	SNP analyzed in replication	R^2	PROSPER/PHASE			CARE (n=711)			WOSCOPS (n=522)		
				Beta	SE	p-value	Beta	SE	p-value	Beta	SE	p-value
1	rs12725107	rs17443102*[1]	0.961	0.57	0.14	4.50E-05	-0.09	0.13	0.507	-0.15	0.17	0.372
2	rs1913896	rs1196160	1	-0.58	0.13	1.47E-05	0.03	0.13	0.802	-0.11	0.16	0.490
2	rs17633730	rs17633730		1.49	0.37	6.59E-05	0.05	0.21	0.819	NA		
2	rs453359	rs453359		-0.57	0.14	7.05E-05	0.12	0.10	0.239	0.01	0.15	0.923
2	rs352887	rs352887		-0.43	0.11	9.18E-05	-0.01	0.10	0.888	0.03	0.14	0.822
2	rs17223074	NA		-1.42	0.36	9.60E-05						
2	rs1519483	NA		0.55	0.14	9.67E-05						
3	rs502114	rs9867036	0.817	-0.48	0.11	1.12E-05	-0.04	0.11	0.708	0.09	0.14	0.512
3	rs11709385	rs12019224*[2]	0.841	-0.50	0.12	1.37E-05	0.10	0.10	0.339	-0.05	0.15	0.713
3	rs3172494	rs3172494		-0.64	0.16	9.70E-05	0.00	0.17	0.977	-0.04	0.21	0.854
4	rs12501068	rs12501068		-1.48	0.35	1.98E-05	0.17	0.25	0.504			
5	rs6894567	rs10056599	0.9	-0.61	0.14	1.71E-05	-0.03	0.12	0.775	-0.14	0.17	0.393
5	rs160919	rs160919		-0.52	0.12	2.43E-05	-0.09	0.11	0.452	-0.15	0.16	0.341
7	rs10255565	rs10255565		-0.78	0.18	2.35E-05	-0.03	0.14	0.821	0.03	0.17	0.859
8	rs3934874	rs3934874		-0.68	0.15	4.43E-06	-0.03	0.13	0.847	-0.02	0.17	0.906
8	rs1116816	rs16928045	1	0.52	0.12	2.10E-05	-0.10	0.11	0.356	-0.03	0.16	0.830
8	rs6601319	rs6601319		0.57	0.14	2.73E-05	-0.06	0.11	0.598	NA		
9	rs10971182	rs10971182		1.18	0.27	8.87E-06	0.14	0.20	0.503	NA		
9	rs10907653	NA		-2.68	0.64	2.52E-05						
9	rs6475859	rs7871606	0.917	0.59	0.14	4.70E-05	0.22	0.12	0.063	0.32	0.18	0.069
9	rs7870226	rs7870226		0.45	0.11	7.63E-05	0.05	0.11	0.651	0.09	0.14	0.499
10	rs2791188	rs2791188		0.67	0.15	8.76E-06	-0.01	0.13	0.931	NA		
10	rs868589	rs868589		0.48	0.12	9.71E-05	0.03	0.12	0.825	-0.20	0.16	0.212

Table 3. Replication of most significant loci (p<1E-04) in CARE trial and WOSCOPS study (Continued)

CHR	TopSNP PROSPER/ PHASE	SNP analyzed in replication	R^2	PROSPER/PHASE			CARE (n=711)			WOSCOPS (n=522)		
				Beta	SE	p-value	Beta	SE	p-value	Beta	SE	p-value
11	rs7102569	rs11237851		0.55	0.12	1.65E-06	-0.28	0.11	0.008	-0.17	0.14	0.246
11	rs1079199	rs1079199		0.52	0.12	1.38E-05	0.08	0.12	0.502	-0.02	0.15	0.905
11	rs7927570	rs4944662	0.959	-0.64	0.16	4.80E-05	-0.10	0.13	0.441	-0.04	0.18	0.807
11	rs1263167	rs1263167		0.56	0.14	7.16E-05	0.00	0.13	0.977	NA		
11	rs655130	rs655130		-1.32	0.34	9.43E-05	0.24	0.24	0.312	NA		
12	rs4767452	rs4767452		-0.88	0.21	2.37E-05	0.22	0.16	0.182	0.13	0.24	0.570
12	rs17120361	rs10506392	1	-1.45	0.35	2.88E-05	0.26	0.25	0.296	-0.22	0.34	0.511
12	rs10778228	rs10860977	1	0.48	0.12	4.25E-05	-0.02	0.10	0.836	0.04	0.15	0.789
12	rs7135770	NA		0.79	0.20	8.06E-05						
13	rs9545683	rs9545669	1	-0.75	0.19	7.86E-05	0.12	0.17	0.484	-0.07	0.23	0.769
13	rs1436260	rs1436260		-0.49	0.13	9.76E-05	-0.05	0.12	0.663	0.08	0.16	0.629
14	rs1271562	rs1255641	1	0.77	0.17	9.68E-06	-0.10	0.14	0.472	-0.06	0.22	0.772
14	rs11157718	rs11157718		0.50	0.13	6.92E-05	-0.20	0.12	0.094	0.16	0.17	0.342
14	rs1885155	rs1885155		0.52	0.13	7.55E-05	0.01	0.12	0.937	0.17	0.17	0.317
17	rs11654492	rs11868258	1	-0.46	0.11	3.28E-05	0.18	0.10	0.075	0.17	0.14	0.214
18	rs7242734	rs7242734		-0.84	0.19	7.05E-06	0.31	0.16	0.047	0.09	0.19	0.619
18	rs281018	rs281018		-2.12	0.50	2.58E-05	-0.20	0.18	0.272	0.06	0.24	0.787
18	rs8092360	rs8092360		0.58	0.14	5.12E-05	-0.03	0.13	0.840	0.23	0.18	0.196
19	rs306468	rs306491	0.896	0.47	0.11	2.51E-05	-0.05	0.10	0.593	0.01	0.14	0.949
19	rs271828	rs271828		-0.49	0.12	2.55E-05	-0.11	0.10	0.295	0.30	0.15	0.039

*The PROSPER/PHASE topSNP or a proxy for it was not available in the replication cohorts and the replication for the second topSNP is given, [1]rs11806506 was the second topSNP in PROSPER/PHASE, rs17443102 was used in the replication as a proxy for it, [2]rs12019224 was the second topSNP in PROSPER/PHASE and used in the replication. Abbreviations: CHR, Chromosome; SNP, single nucleotide polymorphism; SE, standard error; NA, not applicable (SNP not available in replication).

We assessed the explained variance in clinical events by our top SNP rs7102569. The Nagelkerke R square of the logistic regression analysis without the SNP and interaction term was 0.020. Including rs7102569 and the interaction term between SNP and pravastatin treatment gave a Nagelkerke R square of 0.028.

In supplementary table 3 we performed a look-up in our GWAS for SNPs previously described in the literature to be associated with a differential event reduction after statin treatment. Only the DNAJC5B SNP rs13279522 was significantly associated with differential event reduction after correcting for multiple testing (p=0.002).

Discussion

This GWAS was set out to assess for clinically meaningful interactions between genetic variants and pravastatin treatment on the incidence of cardiovascular events on a genome-wide level. We did not observe any genetic variant genome-wide significantly associated with a clinically meaningful differential event reduction by pravastatin, taking the power of our study into account ($p<5x10^{-7}$). For loci that had an interaction $p<1x10^{-4}$ in PROSPER, we investigated them further in two independent pravastatin cohorts (CARE and WOSCOPS), which showed no consistent evidence for a significant pharmacogenetic effect for pravastatin.

In the current study, we investigated the interaction between genetic variants and statin treatment on the incidence of cardiovascular events at a GWAS level. Previously the CARE and WOSCOPS trial conducted a GWAS study to identify genetic variants associated with differential CHD event reduction by pravastatin therapy, however for this GWAS a case-only approach was used [16]. CARE and WOSCOPS identified a SNP in the *DNAJC5B* gene associated with a different event reduction by pravastatin therapy. Other studies have investigated variation in clinical events after statin therapy mainly via candidate gene approaches. For example, genetic variations in *CETP* and *APOE* have been suggested to interact with statin treatment on cardiovascular event reduction [24-29]. However most of those results are equivocal and could not be replicated in large meta-analysis or other studies [30-32]. In addition, none of those SNPs were associated with the differential event reduction during statin therapy in our study, not even to a level of $p<1x10^{-4}$.

Although we did not find any genetic variants significantly associated with differential event reduction by pravastatin treatment, the GWAS in the PROSPER/PHASE study should have the statistical power to detect clinically meaningful interactions (interaction effect of 2.0) between genes and treatment at a p-value threshold of $5x10^{-7}$ (80% power). The power to detect smaller but from the

biological point of view perhaps also interesting effects (interaction effect of 1.2), or less common SNPs, was however limited (<10%) and not the purpose of this study. However, we assume that publishing underpowered results, like our SNPs with a MAF<0.25, is not always senseless. Non-significant but promising findings might be indications for further candidate gene studies, or might be useful in larger meta-analysis. Although for the GWAS approach the PROSPER/PHASE study may not be large enough, the PROSPER study is one of the largest studies of this kind, with prospective data on more than 5000 subjects. Also, the mean duration of follow-up was 42 months with virtually no loss to follow-up and an incidence of almost 800 primary events.

A possible limitation of the current study is the replication in the CARE trial and WOSCOPS study. Both studies had performed a case-only approach to investigate the possible interaction between SNP and pravastatin treatment. This approach is valid only if SNP and treatment are independent of each other, but since both CARE and WOSCOPS were a randomized controlled trial they are independent by design.

The aim of pharmacogenetic research is to identify the genetic variants associated with variable drug responses. Finding those genetic variants should lead to improvements in the use of drug therapy through selection of the most appropriate drug based on an individual's genetic make-up [33]. In some areas of disease this has proven to work [34]. For example, genetic variation in the cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase (*VKORC1*) genes explain up to 35% of the variability in required warfarin starting dose[35]. Therefore, since 2010, the United States Food and Drug Administration has required dose recommendations based on *CYP2C9* and *VKORC1* genotypes into the warfarin product label [35]. Another possible area where pharmacogenetics might be of clinical value is antiplatelet therapy [36]. The *COX1 – 842A>G* and *CYP2C19*2* polymorphisms, reported to be associated to aspirin and clopidogrel resistance, are determinants of thrombotic complications in ST-segment elevation myocardial infarction patients treated with percutaneous coronary intervention and seem of clinical significance[37]. With regard to the pharmacogenetics of statin therapy, most GWA studies have focused on the lipid lowering effects of statins. The only genetic variants that have been consistently identified to associate with variation in LDL cholesterol response are within the *APOE* and *LPA* genes[4]. Currently a large meta-analysis is being performed within the Genomic Investigation of Statin Therapy (GIST) consortium, which potentially will identify more loci associated with variation in LDL cholesterol response to statin therapy [4]. A possible target for pharmacogenetic testing in statin treatment is the *SLCO1B1* gene. Variants in this gene were strongly associated with the risk of high

dose simvastatin-induced myopathy and more than 60% of the myopathy cases could be attributed to the minor *SLCO1B1* allele [9]. This SCLCO1B1 variant seems to be only relevant for simvastatin-associated myopathy, and not for subjects treated with other statin types [38]. This implicates that our findings might not be generalizable to other statins. In our pharmacogenetic of pravastatin study however, our most significant SNP rs7102569 explained only less than 1 percent of the variance in clinical events, which seems not clinically meaningful.

Since we were not able to detect a clinically meaningful differential event reduction by any SNP within this relative large sample of 5200 participants and 800 primary events, one might wonder how pharmacogenetics of pravastatin therapy can be of any clinically relevant use for individual patients in clinical care. Therefore we should ask ourselves the question whether personalized medicine should still be an aim of pharmacogenetic research with regard to (prava) statin therapy. With the GWA studies usually common SNPs with small effect sizes are found in relation to the outcome, however exome sequencing may still reveal rare genetic variant of larger effect sizes that can be used in pharmacogenetic research, but these results then would only apply for a small subset of patients. Pooling of more large studies may lead to significant findings. For the pooling of more studies, collaboration in large consortia is necessary. Within the Genomic Investigation of Statin Therapy (GIST) consortium, meta-analysis will be performed to identify possible SNPs associated with differential event reduction after statin therapy. But even when we then will be able to identify associations in such a large consortium, the question remains if those findings are relevant for the individual patient to guide therapy in daily practice. However, the possibility to identify SNPs of lower effect sizes in such meta-analysis may still be beneficial in identifying novel mechanisms/pathways for protection. Alternatively, they could be used to identify potential new drug targets. A possible positive implication of our findings might be that our findings do not suggest any evidence for differential treatment with statins according to genotype sub-groups.

In conclusion, we could not identify genetic variation that was significantly associated at a genome-wide level with a clinically meaningful differential event reduction by pravastatin treatment in a large prospective study. We therefore assume that in daily practice the use of genetic characteristics to personalize pravastatin treatment and to improve prevention of cardiovascular disease will be limited.

Supplementary material

Supplementary material is available at *Athereosclerosis* online.

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CHAPTER 6

Non-response to statin therapy: The importance of distinguishing non-responders from non-adherers in pharmacogenetic studies.

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Abstract

In pharmacogenetic research, genetic variation in non-responders and high-responders are compared with the aim to identify the genetic loci responsible for this variation in response. However an important question is whether the non-responders are true non-responders or whether they actually are non-adherent? Therefore, we describe, within the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) the characteristics of both non-responders and high-responders of statin treatment in order to possibly discriminate non-responders from the non-adherers. Here, we demonstrate that subjects that do not respond to statin therapy are younger ($p=0.001$), more often smoke ($p<0.001$), have a higher alcohol consumption ($p<0.001$), have lower total cholesterol levels ($p<0.001$), have a lower prevalence of hypertension ($p<0.001$), and have lower cognitive function ($p=0.035$) compared to subjects who highly respond to pravastatin treatment. Moreover, we showed that excluding non-responders and/or non-adherers in pharmacogenetic studies provides more robust results, since standard errors are lower. Our results suggest that non-responders to statin therapy are more likely to be non-adherers, since they have more characteristics that we assume to be indicators of high self-perceived health and low disease awareness, making the subjects less adherent to study medication. We suggest that in pharmacogenetic research, extreme non-responders are excluded to overcome the problem that non-adherence is investigated instead of non-responsiveness.

Introduction

Hydroxymethyl-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) are the most commonly prescribed drugs for the prevention of cardiovascular disease worldwide. Statins lower plasma cholesterol levels with 30-50% and are associated with a reduction of cardiovascular events of 20-40% [1]. Statins are generally well tolerated and are believed to have relatively few side effects [2]. However, clinical response is highly variable and not all subjects appear to benefit from statin therapy, only about a third of treated patients achieve the international guideline specified lipid lowering goals [1].

Pharmacogenetic studies aim to find genetic variation that is responsible for the variable response to drug treatment. For that purpose genome-wide genetic variation in high responders and non-responders is usually compared with the aim to identify genetic loci associated with the variation in response [3;4]. Especially in whole genome sequencing studies, only the two extreme phenotypes e.g. the extremely good responders and the non-responders are chosen to reduce costs and enhance efficiency [5]. However, for correct interpretation of this comparison it is essential to be sure that non-responders have actually taken the drug and are not non-responders due to non-adherence.

Pharmacogenetic research is usually best executed in randomized controlled trials, since adherence to medication is closely monitored, by for example, questionnaires, pill count and nowadays electronic medication monitoring devices [6]. However, this monitoring system does not provide certainty that subjects are actually adherent to their medication. Non-adherers can relatively easily work around the control mechanisms, e.g. by discarding drugs before the pill count. Moreover, assessing plasma levels of drugs does not guarantee adherence, apart from the last days before the study blood drawn. In other words, are we capable in discriminating non-responders from non-adherers in pharmacogenetic research? And how should we optimally deal with this problem in pharmacogenetic analyses?

Using data of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) [7;8], we here describe baseline characteristics of differential responder groups to statin treatment in order to find discriminatory factors between likely non-responders and likely non-adherers. Furthermore, we propose how to deal with the misclassification of false non-responders in pharmacogenetic analyses.

Methods

We used data from the PROSPER study [7;8]. In short, the PROSPER study is a prospective multicenter randomized placebo-controlled trial to assess whether treatment with 40 mg daily pravastatin diminishes the risk of major vascular events in elderly. Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or increased risk of such disease because of smoking, hypertension, or diabetes. A total number of 5,804 subjects were randomly assigned to pravastatin (n=2,891) or placebo treatment (n=2,913). At baseline, a brief medical history was taken, vital signs were recorded, and a fasting venous blood sample was collected for biochemical and hematological checks and for lipoprotein quantification. In addition, a Mini-Mental State Examination was conducted to test for cognitive function. Adherence was measured by pill count every three months.

From the pravastatin users (n=2,891), we excluded all subjects who were withdrawn from the PROSPER study in follow-up because they refused study medication or did not attend the follow-up visits (n=346). From the remaining subjects (n= 2,545) the percentage achieved LDL lowering after statin treatment was calculated by taking the mean LDL level for all post statin treatment measurements at month 3, 6, 12, 24 and 36, minus the baseline LDL level, divided by the baseline LDL level and multiplied by 100. If data of one of the measurements for one individual was missing, we took the mean of only the available measurements of that individual as post statin treatment measurement. These data were available for 2,519 subjects.

We then created five groups of achieved LDL lowering (=<10%; 10-20%; 20-30%; 30-40%; >40% LDL lowering) and compared baseline characteristics between these groups. Based on clinical experience, non-responders were defined as =<10% decrease in LDL cholesterol levels and high-responders were defined as >40% decrease in LDL cholesterol levels.

First, we assessed whether there were differences in baseline characteristics between the five groups of achieved LDL lowering using ANOVA. Baseline characteristics included sex, age, education, smoking, alcohol use, BMI, blood pressure, cholesterol level, history of hypertension, diabetes, and vascular disease, and cognitive function. We also assessed differences in baseline characteristics between the non- and high-responders with a student's t-test for continuous variables or the Pearson Chi-square test for categorical variables.

Second, we used binary logistic regression to assess the relative risk of being a non-responder based on the clinical characteristics that were significantly different

between the high and low groups in the first analysis. Continuous measurements were dichotomized based on sex-specific medians. All analyses were adjusted for age and country of origin, and where necessary additionally adjusted for sex. Third, we calculated the number of risk factors per subject and assessed the association between the number of risk factors and non-responder status with binary logistic regression analysis adjusted for age, sex, and country of origin. The sum of the risk scores was not available in seven subjects of the high-responders and in one subject of the non-responders because of missing data of one of the clinical characteristics.

Fourth, we compared the non-adherers based on the pill count with the non-responders based on LDL lowering for baseline characteristics with a student's t-test for continuous variables or the Pearson Chi-square test for categorical variables. Subjects were defined as a non-adherer if they returned more than 18 (20%) pills in the preceding 90 days before their study visit (mean pill count over maximum number of study visits per individual) [9]. Non-responders were those with LDL lowering <10%. There were 24 subjects in both groups, who were excluded from this analysis to facilitate statistical comparison.

Finally, we performed a Genome-Wide Association Study (GWAS) to analyze the genetic variation associated with variation in LDL lowering in all subjects (n=2272) and repeated this analysis with the exclusion of the subjects classified as non-responders (leaving n=2167), with the exclusion of the non-adherers (leaving n=2160) and with the exclusion of both non-responders and non-adherers (leaving n=2078). The total number of subjects is lower in this analysis since the GWAS has not been executed in all PROSPER subjects, since genotyping failed or they were excluded based on the GWAS quality control criteria [10]. No subjects were excluded based on phenotypic outliers. For this analysis, we used 2.5 million imputed SNPs within the PHASE study (the PHArmacogenetic study of Statins in the Elderly) [10]. The analysis was performed with ProbABEL software (http://www.genabel.org/), adjusted for age, sex, and country [11].

Results

Table 1 shows the baseline characteristics of the five groups of percentage LDL lowering after pravastatin treatment. There were significant differences between the groups for sex, current smoker, history of hypertension, age, education, cognitive function, alcohol use, and level of total cholesterol. Moreover, when we compared the baseline characteristics of the 114 non-responders with the characteristics of the 734 high-responders to pravastatin therapy, we found that subjects who did not

respond to pravastatin therapy were by average 1 year younger (p=0.001), more often smoked and drank more alcohol (both p<0.001), had lower total cholesterol levels (p<0.001), had lower prevalence of hypertension (p<0.001), and had lower cognitive function (p=0.035) compared to subjects who highly responded to pravastatin therapy.

Table 1. Association between groups of % LDL lowering to statin treatment and clinical variables

	% LDL lowering in response to pravastatin treatment					
	>40% (n=734)	30-40% (n=989)	20-30% (n=502)	10-20% (n=180)	<=10% (n=114)	P ANOVA
Categorical variables (n, %)						
Females	423 (58)	511 (52)	218 (43)	82 (46)	56 (49)	<0.001
Current smokers	126 (17)	244 (25)	151 (30)	65 (36)	54 (47)*	<0.001
History of hypertension	503 (69)	620 (63)	301 (60)	98 (54)	58 (51)*	<0.001
History of diabetes	79 (11)	104 (11)	58 (12)	16 (8)	7 (6)	0.485
History of vascular disease	335 (46)	437 (44)	228 (45)	77 (43)	46 (40)	0.809
Country:						
Scotland	325 (44)	410 (42)	210 (42)	83 (46)	49 (43)	
Ireland	248 (34)	364 (37)	200 (40)	71 (39)	51 (45)	
The Netherlands	161 (22)	215 (22)	92 (18)	26 (14)	14 (12)	0.253
Continuous variables (mean, se)						
Age (years)	75.7 (0.12)	75.3 (0.11)	75.0 (0.15)	75.1 (0.24)	74.6 (0.29)*	0.001
BMI (kg/m^2)	26.9 (0.15)	26.8 (0.13)	26.9 (0.18)	27.1 (0.33)	26.3 (0.42)	0.433
Education (years)	15.2 (0.08)	15.3 (0.07)	15.3 (0.10)	14.5 (0.11)	15.2 (0.19)	<0.001
MMSE (points)	28.1 (0.06)	28.2 (0.05)	28.0 (0.07)	27.8 (0.12)	27.8 (0.14)*	0.010
Alcohol (units/week)	3.5 (0.29)	5.0 (0.27)	7.2 (0.47)	7.2 (0.80)	6.5 (0.90)*	<0.001
Total cholesterol (mmol/L)	5.9 (0.04)	5.7 (0.03)	5.6 (0.04)	5.4 (0.06)	5.3 (0.08)*	<0.001
SBP (mmHg)	156.0 (0.80)	154.0 (0.70)	155.8 (0.99)	153.4 (1.59)	152.8 (2.14)	0.200
DBP (mmHg)	83.8 (0.41)	83.6 (0.36)	83.6 (0.50)	82.7 (0.83)	83.7 (1.04)	0.828

Abbreviations: BMI, Body Mass Index; MMSE, Mini Mental State Examination; SBP, systolic blood pressure; DBP, diastolic blood pressure.

*significant difference between the groups of <=10% and >40% LDL lowering (all p<0.05)

Next, we calculated the relative risk of being a non-responder for the characteristics that significantly differed between high and non-responders with a binary logistic regression model (table 2). The largest relative risk was found for subjects that were current smokers (OR 3.96, 95% CI 2.60-6.03, p=1.4 x 10^{-10}). We also found a higher risk of being a non-responder in subjects without a history of hypertension (OR 2.01, 95%CI 1.32-3.04, p=0.001), with a lower cognitive function (OR 1.46, 95%CI 0.97-2.20, p=0.068), with higher alcohol intake (OR 1.73, 95%CI 1.15-2.59, p=0.008), and with lower total cholesterol levels (OR 3.12, 95%CI 2.02-4.81, p=2.6 x 10^{-7}). The association between number of characteristics in the non-responders compared to

the high responders is also shown in table 2. Compared to subjects with none or 1 risk factor, the relative risk of being a non-responder increased gradually to 14.66 (95%CI 5.51-39.02, $p=7.6 \times 10^{-8}$) for subjects with 5 characteristics. When the summary score was included in the model as a continuous variable, the risk of being a non-responder increased with 1.99 (95%CI 1.65-2.38, $p=1.7 \times 10^{-13}$) per additional characteristic.

Table 2. Association between baseline characteristics and being a non-responder

	High-responders (n=734)	Non-responders (n=114)	OR (95%CI)*	p-value
Baseline characteristics				
Smoking	126 (17)	54 (47)	3.96 (2.60-6.03)	1.43×10^{-10}
No history of hypertension	231 (32)	56 (49)	2.01 (1.32-3.04)	0.001
Low MMSE	379 (52)	68 (60)	1.46 (0.97-2.20)	0.068
High Alcohol	270 (37)	58 (51)	1.73 (1.15-2.59)	0.008
Low TC	318 (43)	81 (71)	3.12 (2.02-4.81)	2.58×10^{-7}
Number of characteristics				
<=1	297 (41)	20 (18)	1.0 (ref)	-
2	256 (35)	26 (23)	1.53 (0.83-2.83)	0.170
3	126 (17)	36 (32)	4.15 (2.28-7.55)	3.22×10^{-6}
4	38 (5)	20 (18)	7.25 (3.53-14.87)	6.54×10^{-8}
5	10 (1)	11 (10)	14.66 (5.51-39.02)	7.57×10^{-8}
Trend			1.99 (1.65-2.38)	1.65×10^{-13}

*The OR represents the risk of being a non-responder when you are in the risk category.

The continuous factors are dichotomized based on sex-specific medians. Adjusted for age and country, the analyses for smoking and hypertension are additionally adjusted for sex.

Abbreviations: OR, Odds Ratio; TC, total cholesterol; SBP, Systolic blood pressure; MMSE, Mini Mental State Examination.

Based on pill count, we defined a non-adherer if they returned more than 18 (20%) pills in the preceding 90 days before their study visit (mean pill count over maximum number of study visits per individual). Within the subjects that highly respond to pravastatin therapy 99.5% were adherent to their study medication based on pill count, whereas in the non-responders group this was reduced to 78.6%. Table 3 shows the comparison between non-adherers of the PROSPER study based on pill count and the non-responders based on LDL lowering. Compared to the non-adherers, non-responders smoked more often (p=0.085) and had higher alcohol intake (p=0.117), lower total cholesterol levels (p=0.020), lower systolic blood pressure (p=0.034), and had less often a history of hypertension (p=0.001) and diabetes (0.273) although not all comparisons were statistically significant different. A major difference between the two groups was the number of subjects with a history of vascular disease. Within the non-adheres, there were no subjects with a

history of vascular disease whereas in the non-responder group, 46 (51%) had a history of vascular disease (p<0.001).

Table 3. Comparison of baseline characteristics between non-adherers and non-responders

	Non-adherers (n=98)	Non-responders (n=90)	p-value
Categorical variables (n, %)			
Females	57 (58)	41 (46)	0.057
Current smokers	34 (35)	41 (46)	0.085
History of hypertension	72 (74)	45 (50)	0.001
History of diabetes	10 (10)	6 (7)	0.273
History of vascular disease	0 (0)	46 (51)	<0.001
Country:			
Scotland	35 (36)	40 (44)	
Ireland	54 (55)	39 (43)	
The Netherlands	9 (9)	11 (12)	0.270
Continuous variables (mean, se)			
Age (years)	75.4 (0.35)	74.5 (0.33)	0.070
BMI (kg/m^2)	26.8 (0.43)	26.0 (0.48)	0.215
Education (years)	15.2 (0.19)	15.0 (0.20)	0.458
MMSE (points)	27.4 (0.17)	27.8 (0.16)	0.074
Alcohol (units/week)	4.9 (0.84)	7.0 (1.07)	0.117
Total cholesterol (mmol/L)	5.68 (0.10)	5.35 (0.10)	0.020
SBP (mmHg)	157.0 (2.01)	150.5 (2.29)	0.034
DBP (mmHg)	84.54 (1.21)	83.08 (1.15)	0.385

Subjects who were both non-responder and non-adherer were removed from the analysis to facilitate statistical comparison.

Finally, we compared the results of the GWA studies on the influence of genetic variation of the LDL lowering response after pravastatin treatment in all subjects (n=2272) and in the sample excluding non-responders (n=2167), in the sample excluding non-adherers (n=2160) and in the sample excluding both non-responders and non-adherers (n=2078). The results of the GWA studies are depicted in figure 1. None of the Manhattan plots show any genome wide significant results (all p>5.0 x 10^{-8}). From 4 SNPs known to be associated with statin response the results for the four different analyses are compared in table 4. The main message of this comparison is that by excluding non-responders or non-adherers, the standard error decreases, indicating that probably noise is removed from the analysis. The beta stays more or less consistent in the analysis in the three restricted study samples, however since the SE decreases, also the p-value decreases.

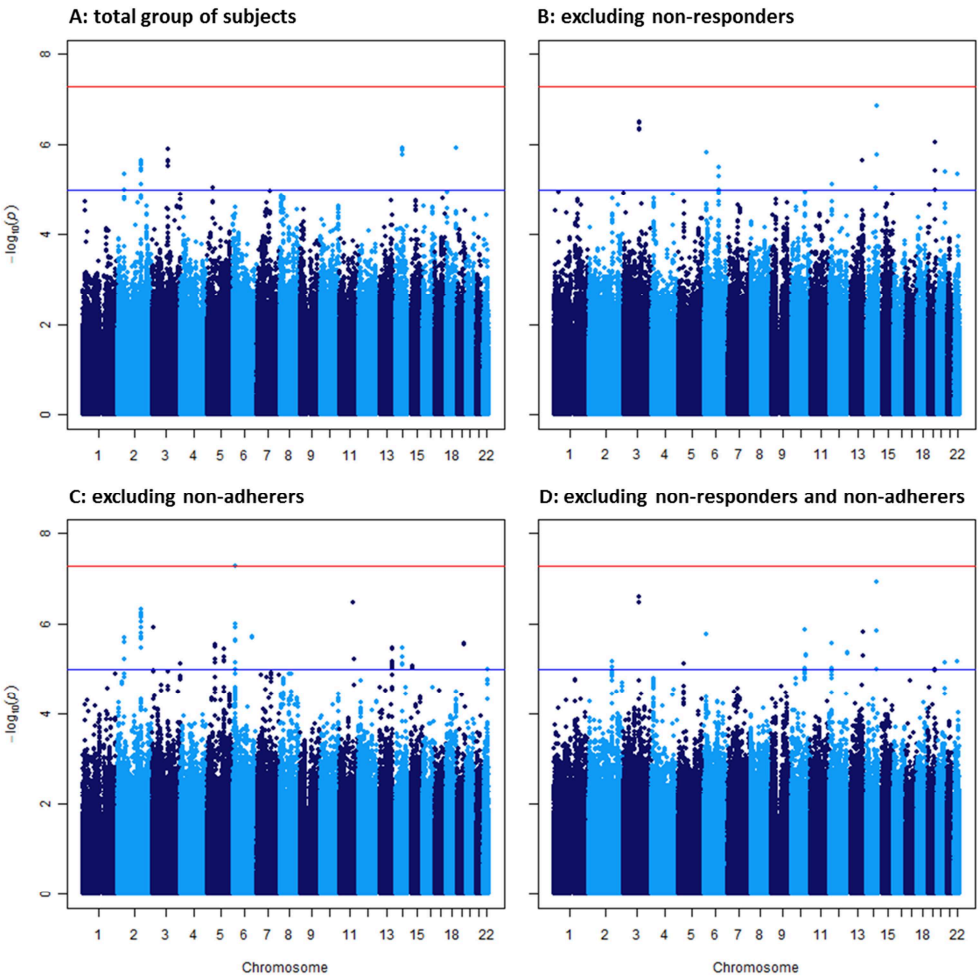

Figure 1. Manhattan plots showing the results of GWA studies on the influence of genetic variation of the LDL lowering response after pravastatin treatment in all subjects (A) and in the sample excluding non-responders (B), in the sample excluding non-adherers (C) and in the sample excluding both non-responders and non-adherers (D).

Discussion

In this study we showed that non-responders to statin treatment differ depending on baseline clinical characteristics from high-responders. Non-responders were more often smokers, drank more alcohol, had a lower cognitive function, were less likely to have hypertension and had lower total cholesterol levels. These characteristics can be considered as indicators of higher self-perceived health and lower disease awareness, indicating that non-responders are less aware of the benefits of using the study medication and are therefore more likely to be non-adherers than non-

responders. Also, compared to the non-adherers based on pill count, non-responders were more likely to be non-adherers since they have more characteristics that correspond with high self-perceived health and low disease awareness. Moreover, we showed that exclusion of the non-responders in the GWAS yielded more robust results, since the standard errors decreased after exclusion. All these results together indicate that pharmacogenetic studies that compare extreme phenotypes might be at least partially biased by the phenomenon of some, perhaps many, non-adherers probably being misclassified as non-responders.

Table 4. Comparison of four SNPs associated with statin response in four different study samples

	N	Beta	SE	p-value
rs10455872 (*LPA*)				
All subjects	2272	-0.0351	0.0123	0.0042
Excl. non-responders	2167	-0.0288	0.0115	0.0122
Excl. non-adherers	2160	-0.0334	0.0124	0.0069
Excl. non-responders + non-adherers	2078	-0.0281	0.0117	0.0162
rs2900478 (*SLCO1B1*)				
All subjects	2272	0.021	0.0065	0.0014
Excl. non-responders	2167	0.020	0.0061	0.0008
Excl. non-adherers	2160	0.022	0.0065	0.0007
Excl. non-responders + non-adherers	2078	0.021	0.0062	0.0006
rs445925 (*APOE*)				
All subjects	2272	0.022	0.0088	0.0121
Excl. non-responders	2167	0.021	0.0082	0.0097
Excl. non-adherers	2160	0.024	0.0090	0.0066
Excl. non-responders + non-adherers	2078	0.024	0.0085	0.0049
rs646776 (*SORT1/CELSR2/PSRC1*)				
All subjects	2272	0.014	0.0058	0.0129
Excl. non-responders	2167	0.016	0.0054	0.0033
Excl. non-adherers	2160	0.018	0.0058	0.0020
Excl. non-responders + non-adherers	2078	0.017	0.0055	0.0020

Only a few studies have investigated differences between non-responders and high-responders of statin therapy [12-15]. Each study showed that characteristics that are indicators of better self-perceived health like age, the number of comorbidities and diet habits are different between non- and high-responders and are therefore more indicators of non-adherence [16;17]. However, we cannot rule out the possibility that these characteristics are actually real factors that determine whether a subject responds biologically different to statin therapy. For example, high-responders of statin therapy have higher baseline cholesterol levels, probably since subjects with higher baseline cholesterol levels could also decrease more in cholesterol level (simply because a greater absolute but also relative change is achievable) after statin therapy compared to subjects with low baseline cholesterol. In this case it is still not

certain if this variable can help us to discriminate between non-responders and non-adherers. However, in various subgroup analyses within the PROSPER study we found no evidence that there is an interaction between any of the clinical characteristics and statin response [8].

The comparison with the actual non-adherers of the PROSPER study based on pill count at each study visit also does not give a conclusive answer. Adherence to study medication in randomized controlled trials like the PROSPER study, is closely monitored by for example questionnaires and by pill count [6]. However, this monitoring system does not provide certainty that subjects are actually adherent to their study medication. Non-adherers can relatively easily work around the control mechanisms, e.g. by discarding a reasonable number of pills before the study visit. Since we showed that the non-responders of pravastatin therapy based on the clinical outcome LDL lowering had more characteristics that we think coincide with high self-perceived health and low disease awareness, we think we have missed non-adherers by using the pill count monitoring system. On the contrary, none of the non-adherers had a history of vascular disease compared to 51% of the non-responders, which indicates that in the non-responder group subjects are included, s.a. those with a history of vascular disease, that likely are adherent and therefore biologically non-responders to the drug.

In many pharmacogenetic studies, non-responders are compared to high-responders to investigate which genetic variation is responsible for this difference in response [5]. However we believe that by using this comparison the best power and most efficiency is reached, there is the possibility that actually the non-adherent phenotype is investigated. Hence, instead of finding genetic variation responsible for the variation in response to therapy, genetic variation for adherence is assessed. Therefore we assessed the difference in analyses when we perform pharmacogenetic research in all subjects compared to pharmacogenetic research excluding the non-responders and/or non-adherers. Our results suggest that in all analyses excluding non-responders and/or non-adherers the noise of the possible non-adherence is reduced since the standard errors were decreased, which cannot be the result of a larger sample size.

Our suggestion is that in pharmacogenetic research, another strategy should be followed to find the genetic variation responsible for the difference in response to (statin) therapy instead of comparing the extreme phenotypes (high- vs. non-responders). We propose three different strategies that may be followed to exclude the problem of investigating non-adherence instead of non-responsiveness. First, all

subjects should be investigated with a total range of responsiveness as a continuous phenotype. In this way the extreme non-responsive cases which are possible non-adherers will not have large weight in the analyses compared to an analysis where non-responders and high-responders are compared. The second proposed strategy is to exclude subjects with non-responsiveness and/or non-adherence and investigate the moderate-responders to the high-responders to be sure that the non-adherence phenotype is excluded from the analysis. And the third, most sophisticated, strategy is to use a propensity score based on various clinical characteristics associated with non-adherence to match high-responders to non-responders. This analysis will exclude any possible confounding from non-adherence from the study. Unfortunately, we could not perform such analysis due to low statistical power.

In conclusion, pharmacogenetic studies that are investigating the difference between non- and high-responders were almost certainly in part investigating the non-compliant phenotype, since non-responders have clinical characteristics that coincide with high self-perceived health and low-disease awareness and that are also very common in non-adherers. Other strategies, as proposed herein, should be used to investigate the relation between genetic variation and responsiveness to (statin) treatment.

Acknowledgements

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CHAPTER 7

Editorial: Statins work around the world

Iris Postmus, Stella Trompet, J Wouter Jukema

Curr Med Res Opin 2013; 29(7):747-749

Cardiovascular disease (CVD) is the leading cause of death worldwide. CVD accounts for 17.3 million deaths per year, a number that is expected to grow to over 23.6 million by the year 2030 [1;2]. During the last decades the CVD death rates have declined in several developed countries, however in low- and middle-income countries the rates of CVD have risen [2;3]. Eighty percent of the CVD deaths worldwide occur nowadays in the low- and middle-income countries, usually at younger age and involve women more frequently [4]. An important risk factor for CVD is hypercholesterolemia. Many studies have proven the effectiveness of lipid-lowering therapy, predominantly statins, in reducing the incidence of CVD [5]. The successfulness of statin therapy is dependent on the decrease in low-density lipoprotein cholesterol (LDLc) [5], therefore achieving the recommended lipid levels is essential in the treatment of CVD.

In this issue of *Current Medical Research and Opinion*, Lee *et al.* investigated the achievement of the recommended LDLc goals and factors associated with this achievement of 1851 Korean patients with hypercholesterolemia treated with rosuvastatin [6]. The LDLc target was defined according to National Cholesterol Education Program – Adult Treatment Panel III guidelines [7], patients were divided into four groups based on their coronary heart disease risk: low, moderate, high, and very high. The LDLc target was dependent on the risk category, < 130 mg/dL for low or moderate risk subjects, < 100 mg/dL for high-risk subjects, and < 70 mg/dL. The attainment rates were relatively high; overall, 88% of the patients reached their LDLc goal. Next, the authors investigated which factors influenced the achieved targets. It was shown that good adherence with medication was a strong predictor of target achievement.

Lee *et al.* have investigated the target achievement in a Korean population, but are there indications that this would be different in other regions of the world? The INTERHEART study was an international case-control study to assess the importance of risk factors for coronary heart disease worldwide [3]. Although the relative importance of every risk factor varied and was dependent on its prevalence, the effect of the risk factors was consistent across different geographic regions and by ethnic background. Since risk factors are comparable around the world, it would seem logical that treatment strategies for the prevention of CVD are also similar in every country. Statins are used worldwide to lower LDLc levels and thereby decreasing CVD risk. But is the success rate comparable in different geographical regions? The Lipid Treatment Assessment Project 2 (L-TAP 2) was a survey performed in nine countries worldwide, and evaluated the proportion of patients achieving the LDLc treatment goals with statins [8]. The proportion of patients attaining the

treatment goal ranged from 47 to 84% across the different countries, with the highest success rate in Korea en the lowest in Spain. The success rate was dependent on the CVD risk, the higher the risk, the lower the achievement of the LDLc goal, being 86% in low-risk patients, 74% in moderate-risk patients, 67% in high-risk patients, and only 30% in the very-high-risk patients, indicating that it is especially difficult to reach the stringent goals in very-high-risk patients.

The slightly observed differences in success rates between countries are likely due to differences in guidelines, patient characteristics, and healthcare systems among the countries. But pharmacokinetic factors may also influence the rate of goal achievement. A study performed in four different ethnic groups living in Singapore investigated the pharmacokinetics of rosuvastatin [9]. Plasma exposure of rosuvastatin and its metabolites was significantly higher in the Asian groups compared with the white subjects. This higher plasma exposure in Asian subjects might be associated with the higher LDLc goal achievement in Korean subjects as observed in the study of Lee *et al.* and L-TAP 2 [6;8]. Differences in genetic background may also be associated with differences in statin response. For example, the occurrence of genetic risk variants or the allele frequencies can be different across populations [10]. An example of racial differences in statin pharmacogenetics was shown in the Cholesterol and Pharmacogenetics (CAP) study. In the CAP study the association between haplotypes in the Low-density lipoprotein receptor (LDLR) gene and lipid-lowering response to simvastatin was assessed in blacks and whites [11]. The *LDLR* haplotype 5 was associated with smaller LDLc reduction in black but not in the white participants. Recent genome-wide association studies (GWAS) have investigated genetic variants associated with the lipid lowering effects of statins, in which the Lipoprotein(a) and apolipoprotein E genes revealed to be associated with statin response [12]. Another study has investigated genetic variants associated with differential coronary heart disease event reduction after statin therapy [13]. However, until now, the majority of the GWAS studies were performed in Caucasian participants [14]. Since there are differences in genetic background, findings from the current GWA studies may not be generalizable to non-Caucasian patients.

Although there seem to be slight differences in goal achievement rates between ethnicities, overall the achievement rates are reasonable, especially in low-risk patients. The overall success rate in the L-TAP 2 survey was 73%, and even 88% in the study by Lee *et al.* [6;8]. The JUPITER (Justification for Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin) trial assessed if there were ethnical differences in the effectiveness of rosuvastatin in reducing first-ever cardiovascular

events [15]. In this trial rosuvastatin was similarly effective in reducing events among whites and nonwhites, including blacks, Hispanics and Asians.

To summarize, statins are used worldwide as lipid-lowering therapy in the prevention of CVD. Despite the many different ethnicities worldwide, the most important risk factors for CVD are for all populations the same. Furthermore, statins seem on average to be effective in all studied populations and thus seem to work all around the world.

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CHAPTER 8

LDL cholesterol still a problem at old age? A Mendelian randomization study

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Abstract

Observational studies in older subjects have shown no or inverse associations between cholesterol levels and mortality. However, in old age plasma low density lipoprotein cholesterol (LDL-C) may not reflect the life-time level due to reverse causality and hence the risk may be underestimated. In the current study, we used an LDL genetic risk score (GRS) to overcome this problem. A weighted GRS was created using 37 single nucleotide polymorphisms associated with LDL-C levels. The LDL GRS was calculated in three Dutch cohorts, the Leiden Longevity Study (LLS) (n=3282), the Leiden 85-plus study (n=317), and the Rotterdam Study (n=4035). We assessed the association between the LDL GRS and LDL-C levels, chronological age, familial longevity, and mortality. In all age strata, individuals with high LDL GRS had higher LDL-C levels (p=0.012 to $p=8.5x10^{-15}$). The frequency of LDL increasing alleles decreased with increasing age (β=-0.027 (SE=0.01) per year, p=0.010). Moreover, individuals with a genetic predisposition for longevity had significantly lower LDL GRS compared to age-matched individuals of the general population (LLS nonagenarians versus >90 years: β=0.93 (SE=0.39), p=0.018, LLS offspring versus partners: β=0.23 (SE=0.10), p=0.019). In longitudinal analysis, high GRS was associated with increased all-cause mortality in individuals >90 years; with a 19% increased risk in individuals with the highest LDL GRS (p-trend=0.008). Results of the current study indicate that a genetic predisposition to high LDL-C levels contributes to mortality throughout life, also in the oldest old and a beneficial LDL genetic risk profile is associated with familial longevity.

Introduction

Observational studies including middle-aged individuals have shown a positive association between cardiovascular disease and cholesterol levels.[1;2] In addition, lowering cholesterol levels with statins reduces the risk of cardiovascular disease at all ages.[1] However, at older ages above 75 years, the contribution of high cholesterol as a cardiovascular risk factor is controversial. Mortality from disease in old age has been shown to be independent of total cholesterol and low-density lipoprotein cholesterol (LDL-C) levels [3;4], whereas low total cholesterol levels have been associated with higher all-cause mortality in the oldest old.[3;5;6] At old age, LDL-C levels in plasma may not reflect life-time LDL-C level due to comorbidities.[3] This inverse health relation in old age raises the question whether lipid levels represent causal factors affecting cardiovascular/metabolic health at all ages. Fortunately, the use of genetic variants as an instrumental variable provides a possibility to investigate the associations free of biases such as reverse causality. In recent years, genome-wide association studies (GWAS) have identified several new genetic loci that are associated with lipoprotein levels. The largest GWAS meta-analysis, including 46 studies comprising more than 100,000 individuals, found 95 loci to be associated with cholesterol levels.[7]

Characteristics of lipid metabolism have further been linked to human lifespan regulation by association to familial longevity. For example, offspring of long-lived individuals have larger LDL particle sizes compared to their spouses or age- and lifestyle-matched controls.[8-10] Moreover, older people who carry the apolipoprotein E gene ε3/ε3 variant and have lower plasma levels of apoE, have a decreased mortality risk compared to carriers of the ε3/ε3 variant with high levels .[11]

In this study we created a genetic risk score (GRS), based on single nucleotide polymorphisms (SNPs) associated with LDL-C levels.[7] Using this GRS as instrumental variable, we evaluated the association between LDL-C and mortality in participants of three Dutch studies (figure 1). In addition, we assessed the association between the LDL GRS and familial longevity.

Methods

Study populations

To assess the associations between the LDL GRS and the various outcomes we made use of three Dutch cohort studies including 7634 participants, the Leiden Longevity

Study (LLS), the Leiden 85-plus study, and the Rotterdam Study. All cohorts had GWA data available and are briefly described here.

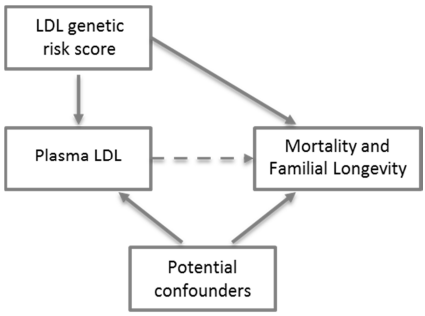

Figure 1. Graphical presentation of the research question in the current study

Leiden Longevity study

For the LLS, long-lived siblings of European descent were recruited together with their offspring and the spouses of the offspring (partners). Families were included if at least two long-lived siblings were alive and fulfilled the age criterion of 89 years or older for men and 91 years or older for women, representing less than 0.5% of the Dutch population in 2001.[12] In total, 931 long-lived siblings with a mean age of 94 years (range 89-104), 1671 offspring (mean age 61 years, range 39-81), and 744 partners (mean 60 years, range 36-79) were included. DNA from the participants of the LLS was extracted from samples at baseline using conventional methods. [13]

Leiden 85-plus study

Participants of the Leiden 85-plus study were inhabitants of Leiden, the Netherlands, who reached the age of 85 years between September 1, 1997, and September 1, 1999. There were no selection criteria on health, functioning or demographic characteristics. A total of 705 inhabitants reached the age of 85 years and a total of 599 individuals participated.[14] Individuals were visited at their place of residence and annual follow up visits were performed until death or age 90 years. Information about mortality was available until December 31, 2009. The date of death was obtained from the civic registry of Leiden.

Rotterdam Study

The Rotterdam Study is a population-based cohort study, including 7983 participants living in Ommoord, a district of Rotterdam, the Netherlands. All inhabitants aged 55 and over, were invited to participate in the study (n = 10275). The Rotterdam Study started in the early 1990s and periodical examinations were performed every three to five years. Analyses of this study are based on data from the third round of the

study which was performed between 1997-1999 (n=4035). The study was approved by the medical ethical committee of the Erasmus Medical Center and written informed consent was obtained from all participants.[15]

Lipoprotein levels

In offspring and partners from the LLS non-fasting venous blood samples were taken. Total and HDL cholesterol levels were determined using fully automated equipment (the Hitachi Modular or the Cobas Inergra 800 both from Roche, Almere, the Netherlands).

In the Leiden 85-plus study lipoprotein levels were obtained at the follow-up visit at age 90 years. Total cholesterol, triglyceride, and HDL cholesterol levels were analysed with fully automated computerized analyzers (Hitachi 747 and 911, Hitachi, Ltd, Tokyo, Japan).

In the Rotterdam Study, total cholesterol, HDL cholesterol, and triglyceride concentrations were measured from serum or plasma extracted from whole blood, using an automated enzymatic procedure (Boehringer Mannheim System).

In all three cohorts, LDL-C levels were calculated using the Friedewald equation.[16]

Genotyping

In the Leiden Longevity study genotyping was performed with Illumina Human660W-Quad and OmniExpress BeadChips (Illumina, San Diego, CA, USA). Individuals were removed if they showed a mismatch in gender or familial relatedness based on genotype and phenotype, leaving 931 nonagenarians, 1610 offspring and 741 partners for the analysis. In addition, SNPs which were not measured on both platforms and with a call rate <0.95, MAF <0.01 and $P_{HWE} < 10^{-4}$ were excluded, leaving 288635 (nonagenarians) and 298538 (offspring and partners) SNPs as input for the imputation. Imputation was performed separately for the LLS nonagenarians and LLS offspring and partners using IMPUTE2 with reference HapMap Phase I + II CEU release 22 (hg18/build36).

In the Leiden 85-plus study genotyping was performed with Illumina OmniExpress BeadChips (Illumina) in participants aged 90 years. Individuals were removed if they showed a mismatch in gender based on genotype and phenotype, leaving 317 individuals for the analysis. In addition, SNPs with a call rate <0.95, MAF <0.01 and $P_{HWE} <10^{-4}$ were excluded, leaving 603301 SNPs as input for the imputation. Imputation was performed using IMPUTE2 with reference HapMap Phase I + II CEU release 22 (hg18/build36).

In the Rotterdam Study genotyping was conducted using the Illumina Infinium II HumanHap 550K array among self-reported Caucasian individuals. Individuals were excluded if they had excess autosomal heterozygosity, mismatch between called and phenotypic gender, or recognized as being outlier with IBS clustering analysis. In addition, SNPs with a MAF ≤1%, P_{HWE} <10^{-5}, or call rate ≤90% were excluded, leaving 530683 SNPs. Imputation was performed using the maximum likelihood method implemented in MaCH (version 1.0.15) with reference to HapMap Phase I + II CEU release 22 (hg18/build36).

Weighted genetic risk score

To create the LDL GRS we used the SNPs identified in the GWAS meta-analysis reported by Teslovich *et al.*.[7] We included all 37 SNPs associated with LDL-C levels (and possibly with total cholesterol, HDL cholesterol, and/or triglycerides).To build the LDL GRS, we first determined the number (or dosage in case of imputed SNPs) of unfavourable alleles for each individual, whereby the unfavourable allele was associated with higher LDL-C levels in the GWAS meta-analysis.[7] The number of unfavourable alleles was multiplied by the absolute effect size as published in the original paper.[7] Next, we calculated the GRS for each individual by summing the estimates (number of unfavourable alleles x absolute effect size) of all SNPs and divided it by the average of all effect sizes. In the final step the GRS was rescaled into a percentage of the maximum number of risk alleles (individuals GRS / maximum GRS score) x 100%. To use the GRS as a categorical variable, the GRS % was divided into three groups, using 47.5% and 52.5% as cut off values.

Statistical analysis

First, to assess the association between LDL GRS categories and LDL-C levels we combined the data of general population subjects (LLS partners, Leiden 85-plus study, and Rotterdam Study) and divided the individuals in age strata of 10 years. Data of LLS nonagenarians and offspring were excluded from this analysis since they have a genetic predisposition for longevity and to exclude possible familial effects. A general linear model was used adjusted for age, sex, and cohort. The explained variance in LDL-C levels by the LDL GRS was assessed by calculating the R^2 per cohort using a linear regression model.

Second, we assessed the cross-sectional association between the LDL GRS and chronological age. Individual level data from the LLS partners, Leiden 85-plus study, and the Rotterdam Study were combined to have a wide variation in age range. A general linear model was used adjusted for sex and cohort. Additional analyses were performed using only the individuals aged ≥ 50 years, and ≥70 years.

Differences in LDL GRS between LLS nonagenarians and individuals ≥90 years, and LLS partners and offspring were tested using a general linear model adjusted for age, sex, and, if necessary, familial relations.

Finally, the longitudinal association between LDL GRS categories and mortality in individuals ≥90 years was assessed using Poisson analysis to calculate incidence rate ratios. For this analysis, data of the LLS nonagenarians, Leiden 85-plus study and Rotterdam Study participants aged ≥90 years was used. Incidence rate ratios were adjusted for age, sex, and, if necessary, cohort and familial relations.

All statistical analyses were performed using IBM SPSS Statistics program for Windows (Version 20.0, USA) and Stata/SE version 12.1 for Windows. P-values ≤0.05 were considered statistically significant.

Results

The LDL GRS was calculated for 3282 participants (931 nonagenarians, 1610 offspring, and 741 partners) of the Leiden Longevity Study, 317 participants of the Leiden 85-plus study, and 4035 participants of the Rotterdam study. In table 1, the baseline characteristics are shown for the six age strata including the general population participants (LLS partners, Leiden 85-plus study, and Rotterdam Study), the LLS nonagenarians and offspring. Baseline characteristics per cohort are provided in supplementary table 1.

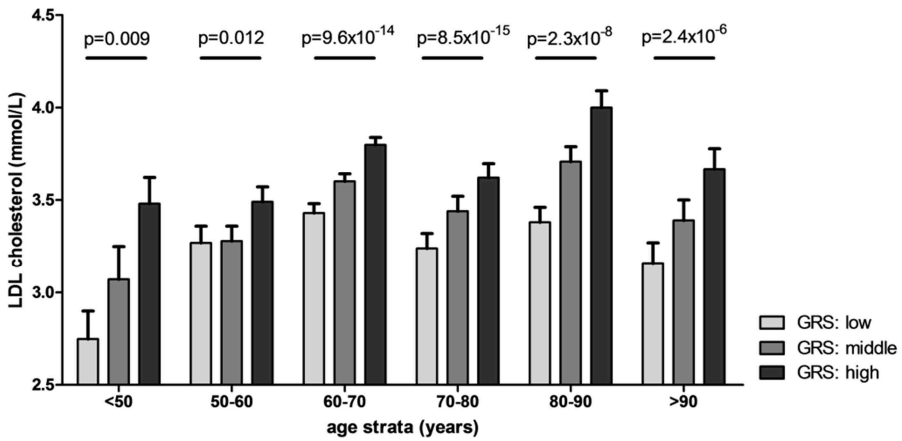

Figure 2: LDL cholesterol levels per GRS category in different age strata, using individual level data of LLS partners, Leiden 85-plus study, and the Rotterdam Study. Means and standard errors (SE) were assessed using a general linear model adjusted for age, sex, and cohort. P-values were assessed using the continuous values of the LDL GRS.

Table 1. Baseline characteristics

	Age strata (years)						Leiden Longevity study	
	<50	50-60	60-70	70-80	80-90	>90	Offspring	Nonagenarians
N	88	327	1859	1739	655	425	1610	931
Age (years)	45.8 (4.3)	55.7 (2.6)	65.7 (2.5)	74.7 (2.9)	83.9 (2.7)	90.8 (2.1)	59.4 (6.5)	93.4 (2.7)
Male (%)	25 (28.4)	108 (33.0)	834 (44.9)	785 (45.1)	199 (30.4)	113 (26.6)	747 (46.4)	350 (37.6)
Body mass index (kg/m^2)	24.5 (3.3)	25.8 (3.8)	26.7 (3.9)	26.9 (3.9)	26.6 (4.0)	25.8 (4.4)	25.3 (3.6)	25.5 (3.6)
Total cholesterol (mmol/L)	5.33 (1.02)	5.67 (1.02)	5.85 (1.00)	5.77 (0.97)	5.77 (1.09)	5.22 (1.04)	5.59 (1.12)	NA
LDL cholesterol (mmol/L)	3.12 (0.83)	3.36 (0.86)	3.73 (0.91)	3.69 (0.87)	3.67 (1.00)	3.25 (0.89)	3.32 (0.93)	NA
HDL cholesterol (mmol/L)	1.46 (0.44)	1.45 (0.47)	1.38 (0.38)	1.38 (0.40)	1.43 (0.44)	1.36 (0.42)	1.45 (0.44)	NA
Triglycerides (mmol/L)	1.61 (1.11)	1.75 (1.05)	1.67 (0.88)	1.55 (0.80)	1.50 (0.70)	1.41 (0.84)	1.76 (1.03)	NA
Cohort (%)								
LLS Partners	88 (100)	327 (100)	284 (15.3)	42 (2.4)	0 (0)	0 (0)		
Leiden 85-plus study	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	317 (74.6)		
Rotterdam Study	0 (0)	0 (0)	1575 (84.7)	1697 (97.6)	655 (100)	108 (25.4)		

Continuous variables are presented as means and standard deviations, categorical variables are presented as numbers and percentages.

Table 2. Association between LDL genetic risk score categories and mortality in participants aged ≥90 years

	Genetic risk score category								
	Low		Middle		High		IRR (95% CI)		
	Deaths	PY (x1000)	Deaths	PY (x1000)	Deaths	PY (x1000)	2 vs 1	3 vs 1	P-trend
Leiden 85-plus study	89	0.805	82	0.755	97	0.785	0.98 (0.72-1.32)	1.11 (0.84-1.49)	0.445
Rotterdam Study	31	0.122	37	0.160	25	0.111	0.95 (0.55-1.66)	0.98 (0.54-1.78)	0.962
LLS Nonagenarians	294	1.309	251	1.039	199	0.738	1.07 (0.90-1.27)	1.25 (1.04-1.49)	0.010
Combined	414	2.235	369	1.950	321	1.634	1.04 (0.91-1.18)	1.19 (1.05-1.34)	0.008

Incidence rates and incidence rate ratios are shown with their 95% confidence interval. Incidence rate ratios were assessed using Poisson analysis, adjusted for age, sex, cohort, and were necessary familial relations.
Abbreviations: PY, person years; IR, incidence rate; IRR, incidence rate ratio; CI, confidence interval; vs, versus; LLS, Leiden Longevity Study.

In each age stratum there was a linear association between the LDL GRS and LDL-C levels (figure 2), with individuals in the highest LDL GRS group having the highest LDL-C level (p=0.012 to $p=8.5x10^{-15}$). Associations between the LDL GRS and LDL-C levels in the separate cohorts are provided in supplementary figure 1, showing a linear association in each cohort. The LDL GRS explained 7.1% of the variance in LDL-C levels in the LLS offspring, 4.4% in the LLS partners, 6.0% in the Leiden 85-plus study, and 3.8% in the total Rotterdam Study.

The next step was to assess the association between the LDL GRS and chronological age. Figure 3 shows the cross-sectional relation between the LDL GRS and age. With increasing age, the LDL GRS decreased. Regression analysis showed a significant association between LDL GRS and chronological age (β=-0.27 (SE=0.1) per 10-year increase in age, p=0.010, adjusted for sex and cohort). Additional separate analyses were performed in participants aged ≥50 years and in participants aged ≥70 years. In both age groups the significant association between the LDL GRS and age remained when excluding the younger participants (≥50 years: β=-0.26 (SE=0.11) per 10-year increase in age, p=0.015; ≥70 years: β=-0.52 (SE=0.18) per 10-years increase in age, p=0.005).

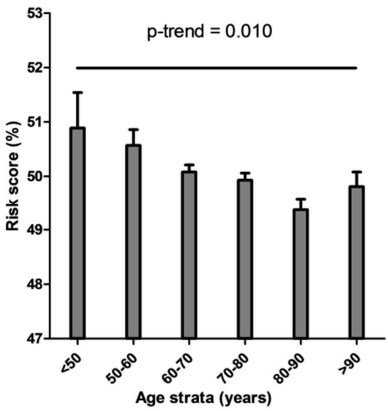

Figure 3: Mean percentage of LDL GRS for each age category, including LLS partners, participants of the Leiden 85-plus study, and the Rotterdam Study. The LDL GRS is plotted as mean percentage with standard error. P-value calculated for the association between age and LDL GRS, adjusted for sex and cohort.

Next, we investigated if the LDL GRS was associated with familial longevity. For this purpose, we compared the mean LDL GRS in the LLS nonagenarians versus the individuals aged ≥90 years from the Leiden 85-plus study and the Rotterdam Study (figure 4A), and in the LLS offspring versus the LLS partners (figure 4B). The mean

GRS was significantly lower for individuals with a predisposition for longevity compared to the individuals from the general population within the same age range. LLS nonagenarians had a mean LDL GRS of 48.8% (SD=0.2) compared to a mean LDL GRS of 49.7% (SE=0.3) for the individuals aged ≥90 years from the general population ($p_{difference}$=0.018). LLS offspring had a mean LDL GRS of 50.0% (SE=0.1), while the partners had a mean LDL GRS of 50.7% (SE=0.2) ($p_{difference}$=0.019).

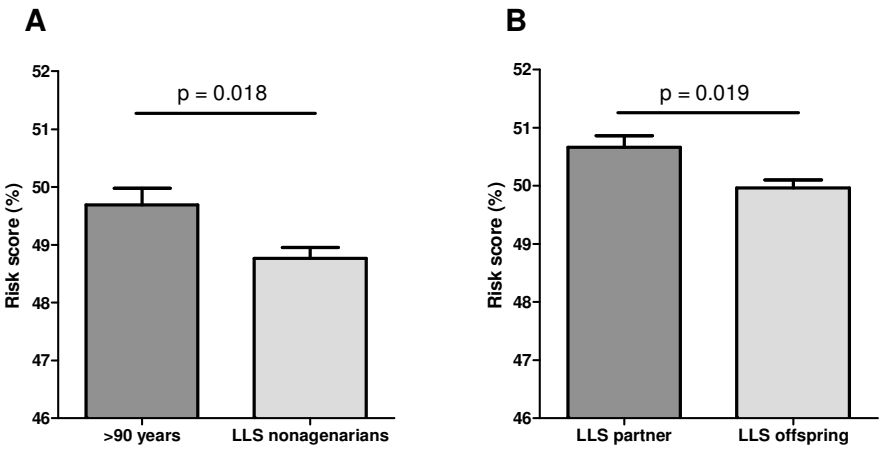

Figure 4: Mean percentage of LDL GRS in participants of the general population (dark grey) and genetically enriched for longevity (light grey). A: Subjects aged ≥90 years from the Leiden 85-plus study and Rotterdam Study versus Leiden Longevity study (LLS) nonagenarians; B: LLS partners versus offspring. GRS plotted as mean with standard error, adjusted for age, sex, and familial relations.

Finally, we investigated the association between the LDL GRS and mortality in the elderly. For this purpose, we used the data of the LLS nonagenarians and of participants aged ≥ 90 years from the Leiden 85-plus study and the Rotterdam Study. The combined analysis of the three studies showed a significant association between the LDL GRS and increased all-cause mortality (table 2). Individuals in the middle LDL GRS group had 4% increased mortality risk (IRR 1.04, 95% CI: 0.90-1.19), and individuals in the highest group had 19% increased mortality risk (IRR 1.19, 95% CI: 1.02-1.37), compared to individuals in the lowest LDL GRS group (p-trend=0.008). Analyses in the individual studies showed a significant association between LDL GRS and mortality in the LLS nonagenarians (p=0.010), with 25% increased mortality risk (95% CI: 1.04-1.49) for individuals in the highest LDL GRS group, compared to individuals in the lowest group. Within the Leiden 85-plus study and the Rotterdam Study, the LDL GRS was not significantly associated with mortality (p=0.445 and p=0.962, respectively).

Discussion

In old age, the importance of high LDL-C levels as risk factor for mortality is unclear since observational studies have shown no or inverse associations. Due to confounding or reversed causality, the plasma LDL-C levels may not reflect the life-time level. To overcome the potential influences of reverse causality and confounding we used in the current study the LDL GRS as an instrumental variable. The LDL GRS was strongly associated with LDL-C levels and the number of LDL increasing alleles decreased with increasing age. Furthermore, individuals with a genetic predisposition for longevity had a lower LDL GRS compared to age-matched controls. Finally, we showed that the LDL GRS was associated with all-cause mortality above 90 years in the pooled analysis of three independent populations, although this effect was mainly driven by one study. All these results indicate that a genetic predisposition to high LDL-C levels contributes to mortality throughout life, also in the oldest old, and a beneficial LDL genetic profile is associated with familial longevity.

Observational studies have repeatedly shown a positive association between high cholesterol levels and increased mortality risks.[17] However, it is unclear whether this positive association remains in the elderly. Several studies in people aged 80 years and over showed an association between low total cholesterol levels and increased mortality.[5] Previously reported analysis of the Leiden 85-plus study did not observe any association between high LDL-C levels and mortality, and high total cholesterol levels were associated with longevity.[3;4] Cholesterol levels of elderly aged 85 years and over might not reflect their life-time cholesterol level, due to reverse causality and possible selective survival.[3] Our results using the LDL GRS as an instrumental variable indicate that the results from observational studies in elderly using plasma cholesterol as a reflection of risk were probably biased.

The observed association between the LDL GRS and mortality was only significant in the LLS siblings and the combined analysis. This might be explained by the lower number of individuals aged 90 years and over in the Leiden 85-plus and Rotterdam study compared to the LLS siblings. We did observe that the level of LDL GRS decreased by increasing age, this was however not reflected in all prospective studies. A similar phenomenon was observed earlier for the *APOE* gene. A lower frequency of the APOE ε4 allele with increasing age was reported already in 1988.[18] However, associations between the *APOE* gene and mortality were reported since 1994 in large studies. [19;20] Nowadays, the association between genetic variation in

the *APOE* gene and longevity has repeatedly been validated in large prospective studies with sufficient statistical power. [20;21]

Genetic risk scores based on SNPs associated with cholesterol levels have been used previously. Within the CARDIoGRAM consortium, including more than 53146 myocardial infarction cases and controls, the association between cholesterol levels and the risk of myocardial infarction (MI) was compared to the association between GRS and the risk of MI.[22] An increase in both plasma LDL-C levels as well as LDL-C conferred by the GRS was associated with an increased risk of MI. Increased HDL cholesterol was associated with a decreased risk for MI, although the HDL GRS was not associated with the risk for MI, indicating that HDL cholesterol is not a causal risk factor for MI.[22] Recently, a GRS based on LDL-C SNPs was tested in two British prospective studies, including middle-aged men and women.[23] Participants in the top quintile of the genetic score distribution tended to have a 36-49% increased risk of having a high CVD risk, determined by the Framingham 10-year CVD risk more than 20%, compared to individuals in the lowest quintile. Our study shows that an association with mortality is still present at old age.

In the current study we observed a difference in LDL GRS between offspring of nonagenarians and their spouses and between LLS nonagenarians and individuals aged ≥90 years. Individuals with a genetic predisposition for longevity had a lower LDL GRS, indicating the beneficial effects of low LDL cholesterol levels. This finding is the first difference in genetic risk scores observed between the LLS offspring and partners. Previous studies found a lower prevalence of diabetes mellitus, hypertension, and myocardial infarction in LLS offspring compared to their partners.[24] Furthermore, the offspring had a more beneficial metabolic profile.[25] A GRS based on diabetes risk alleles has previously been tested in the LLS partners and offspring, and despite the better glucose tolerance of the offspring, this was not associated with differences in GRS.[25]

To summarize, previous observational studies including older individuals have shown no or inverse associations between cholesterol levels and mortality, suggesting that the causal relation between LDL and (cardiovascular) disease is absent at old age. Results of the current study indicate that a genetic predisposition to high LDL-C contributes to mortality throughout life, also in the oldest old and a beneficial LDL genetic risk profile is associated with familial longevity.

Supplementary material

Supplementary material is available upon request.

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CHAPTER 9

PCSK9 SNP rs11591147 is associated with low cholesterol levels but not with cognitive performance or noncardiovascular clinical events in an elderly population

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Abstract

Proprotein convertase subtilisin like/kexin type 9 (PCSK9) is a protein involved in the LDL-cholesterol metabolism. The single nucleotide polymorphism (SNP) rs11591147 has been associated with lower LDL-cholesterol levels and a lower risk of coronary heart disease. Since PCSK9 has high affinity to the LDL receptor, inhibiting PCSK9 is a testable therapeutic target for lipid-lowering therapy. Currently, several approaches to inhibit PCSK9 are under development, but it is unknown what the effects of those inhibitors will be on cognition or noncardiovascular clinical events. In this study we assessed the association between rs11591147 and cognitive performance, activities of daily living (ADL), and noncardiovascular clinical events within 5777 participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER). Rs11591147 was associated with 10 to 16% lower LDL-cholesterol levels ($p=3.62 \times 10^{-12}$), but was not associated with cognitive performance, ADL, or noncardiovascular clinical events in the PROSPER study. Our findings suggest that lower cholesterol levels due to genetic variation in the *PCSK9* gene are not associated with cognitive performance, functional status, or noncardiovascular clinical events.

Introduction

Elevated plasma concentration of low-density lipoprotein (LDL)-cholesterol is a major causal risk factor for cardiovascular disease. Lowering LDL-cholesterol levels is one of the primary goals of therapy in its prevention and treatment [1]. Present lipid lowering therapy mainly depends on treatment with HMG-CoA reductase inhibitors (statins), but new lipid lowering drugs are under development that can decrease circulating lipid levels even more [2]. A promising new therapeutic target for lipid-lowering therapy is proprotein convertase subtilisin-like/kexin type 9 (PCSK9), a protein involved in LDL-cholesterol metabolism [3-5]. PCSK9 modulates plasma LDL-cholesterol levels by promoting the degradation of LDL receptor (LDLR) [6].

Several "loss of function" and "gain of function" mutations have been described in the PCSK9 gene [4]. Within the Atherosclerosis Risk in Communities (ARIC) Study "loss of function" mutations, for example rs11591147, were associated with lower LDL-cholesterol levels through increased LDLR levels, resulting in a lower prevalence of peripheral arterial disease, and a reduced risk of coronary heart disease [7;8]. A recent meta-analysis showed 12% lower LDL-cholesterol levels and 28% lower ischemic heart disease risk among carriers of the rs11591147 mutation in the PCSK9 gene [9].

On the other hand, PCSK9 may be involved in processes associated with neurodegenerative disorders like Alzheimer's disease. A Canadian study found a two-fold increased expression of the PCSK9 in the human frontal cortex of autopsy-confirmed Alzheimer's disease cases compared with age-matched controls [10]. Furthermore, men carrying either the rs2483205-C, rs483462-A, or rs662145-T allele showed a significant increased risk for Alzheimer's disease. However, those single nucleotide polymorphisms (SNPs) are not known to be associated with LDL-cholesterol levels or with cardiovascular disease risk.

Several approaches to inhibit PCSK9 are currently under development, but it is not known whether those inhibitors will influence cognitive performance or clinical events. In old age, cholesterol levels are not associated with cognitive performance, and it has previously been shown in the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER), that pravastatin therapy in old age did not affect cognitive decline over a median follow-up of 3.2 years [11;12]. However, to investigate whether lipid lowering via PCSK9 inhibition might have other clinical effects besides lipid lowering, we assessed the relation between lower LDL levels associated with genetic variation within the *PCSK9* SNP rs11591147 and cognitive function, ADL, and

noncardiovascular clinical events in an elderly population where these events occur frequently.

Methods

Study population

All data come from the PROSPER study. Detailed descriptions of PROSPER have been published elsewhere [13;14]. Briefly, PROSPER was an investigator-driven, prospective multi-national randomized placebo-controlled trial to assess whether treatment with pravastatin reduces the risk of major vascular events in the elderly. Between December 1997 and May 1999, subjects in Scotland (Glasgow), Ireland (Cork) and the Netherlands (Leiden) were screened and enrolled. Men and women aged 70-82 years were recruited if they had pre-existing vascular disease or were at increased risk for such disease because of smoking, diabetes, or hypertension. A total number of 5804 subjects were randomly assigned to pravastatin or placebo. The protocol of the PROSPER study was approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from all participating subjects.

Endpoints

Mean follow-up duration was 3.2 years (range 2.8-4.0) and participants were reviewed every 3 months. During those visits all endpoints were adjudicated by a study Endpoint Committee. The primary outcome in PROSPER was the combined endpoint of definite or suspected death from coronary heart disease, nonfatal myocardial infarction, and fatal or nonfatal stroke. Furthermore several noncardiovascular clinical events were recorded. Cancer incidence was a tertiary study endpoint. Infections and diabetes were reported as serious adverse effects. Since the association between *PCSK9* and cardiovascular events has been investigated previously in PROSPER the focus in the present paper was on noncardiovascular events, functional status, and cognitive performance.

Cognitive performance and activities of daily living (ADL)

The Mini-Mental State Examination (MMSE) was used to measure global cognitive performance. The MMSE scores range from 0 (very severe cognitive impairment) to 30 points (optimal cognitive function). Participants with poor cognitive performance (MMSE <24) were not eligible for inclusion in the PROSPER study. Four neuropsychological performance tests were used to measure various cognitive domains. The Stroop color-word test for attention and the Letter-Digit Coding test (LDT) for processing speed were used to measure executive function. The outcome parameter for the Stroop test was the total number of seconds to complete the third

Stroop card containing 40 items. The outcome variable for the LDT was the total number of correct entries in 60 seconds. Memory was assessed with the 15-Picture Learning test (PLT) testing immediate and delayed recall. The main outcome parameters were the accumulated number of recalled pictures over the three learning trials and the number of pictures recalled after 20 minutes. To assess change of functional status, the 20 point Barthel and Instrumental Activities of Daily Living (IADL) questionnaires, using a modified version of the OARS questionnaire, were performed. The Barthel index is a measure of basic ADL and the IADL measures extended ADL. The reliability and sensitivity of these tests in an elderly population have been published elsewhere [15].

Cognitive performance and ADL was tested at six different time-points during the study, before randomization, at baseline, after 9, 18 and 30 months, and at the end of the study. The time point of this last measurement was between 36 and 48 months and different for the participants, therefore we performed the analyses with their individually varying time-point but report the results for the mean of these time-points (42 months). The pre-randomized measurement was discarded in the analysis to preclude possible learning effects.

Genotyping

Rs11591147 of the PCSK9 gene was genotyped previously in PROSPER using Taq Man® SNPs genotyping assay (Applied Biosystems, Foster City, CA, United States). A detailed description of the genotyping has been published elsewhere [16]. Rs11591147 genotyping succeeded in 5777 of the 5804 PROSPER participants. ApoE phenotype was determined on plasma samples by western blotting, using the method of Havekes et al. [17].

To assess the association between other *PCSK9* SNPs and cholesterol levels, cognitive performance and clinical events we used data of the PHArmacogenetic study of Statins in the Elderly at risk (PHASE), a genome-wide association study (GWAS) in the PROSPER participants [18]. The GWAS was conducted using the Illumina 660-Quad beadchips following manufacturer's instructions. After a stringent quality control 557,192 SNPs were available for analysis. To maximize the availability of genetic data and coverage of the genome, imputation up to 2.5 million autosomal CEPH HapMap SNPs was performed with MACH imputation software based on the Hapmap built II release 23. Detailed descriptions of the PHASE project have been published elsewhere [18]. *PCSK9* SNPs were selected from the GWAS using PLINK software version 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/)[19].

Statistical analysis

Cross-sectional associations between rs11591147 and cognitive function were assessed using a general linear model adjusted for age, sex, country, education, ApoE phenotype and where appropriate version of test used. Repeated cross-sectional associations with cognitive function were assessed with a linear mixed model for repeated measurements and were additionally adjusted for follow-up time. Cross-sectional associations between rs11591147 and history of clinical events were assessed with logistic regression analysis, which was adjusted for age, sex, and country. Longitudinal associations were assessed with Cox regression and were additionally adjusted for pravastatin use. Power calculation was performed using Quanto version 1.2.4, May 2009. The study had 99% power to detect a 2% increased incidence of diabetes by the rs11591147 variant, assuming a 5% incidence rate in the rs11591147 wild-type carriers. PASW statistics software (version 17.0.2, SPSS Inc., Chicago, IL) was used for all statistical analyses. P-values <0.05 were considered statistically significant.

Table 1. Baseline characteristics of PROSPER participants stratified by country

	Scotland (n=2516)	Ireland (n=2166)	the Netherlands (n=1095)
Continuous variables (mean, SD)			
Age (years)	75.3 (3.4)	75.5 (3.3)	75.1 (3.3)
Body mass index (kg/m^2)	26.7 (4.2)	27.0 (4.4)	26.7 (3.8)
Total cholesterol (mmol/l)	5.7 (0.9)	5.6 (0.9)	5.8 (0.9)
LDL-cholesterol (mmol/l)	3.8 (0.8)	3.7 (0.8)	3.9 (0.8)
HDL cholesterol (mmol/l)	1.3 (0.4)	1.3 (0.4)	1.2 (0.3)
Categorical variables (n, %)			
Males	1237 (49.2)	980 (45.2)	577 (52.7)
Current smoker	707 (28.1)	580 (26.8)	267 (24.4)
History of hypertension	1443 (57.4)	1430 (66.0)	703 (64.2)
History of diabetes	213 (8.5)	220 (10.2)	184 (16.8)
History of angina	811 (32.2)	518 (23.9)	222 (20.3)
History of claudication	229 (9.1)	113 (5.2)	46 (4.2)
History of myocardial infarction	379 (15.1)	255 (11.8)	139 (12.7)
History of stroke or TIA	265 (10.5)	222 (10.2)	161 (14.7)
History of vascular disease*	1239 (49.2)	842 (38.9)	473 (43.2)
Genotype, MAF (%)			
Rs11591147 (GT)	2.1	1.8	1.2

* Any of stable angina, intermittent claudication, stroke, transient ischemic attack (TIA), myocardial infarction, peripheral artery disease surgery, or amputation for vascular disease more than 6 months before study entry. Abbreviations: TIA, transient ischemic attack; MAF, Minor allele frequency

Results

Table 1 shows the baseline characteristics of study subjects, stratified by country of origin. The mean age of all subjects was 75.3 years and about 50% of the participants were female. The association between the *PCSK9* SNP rs11591147 and lipoprotein levels and vascular events has previously been described [16]. To summarize these findings, carriers of one rs11591147 variant had 10% lower LDL-cholesterol levels ($p=3.62 \times 10^{-12}$) (Table 2) and a non-significant 9% reduced risk of vascular disease [16].

Table 2 shows the association between rs11591147 and cognitive performance and functional status. There were no associations between rs11591147 and cognitive performance and functional status, either at baseline or during follow-up (all p>0.1).

Table 2. Rs11591147, cholesterol levels, cognitive performance, and functional status at baseline and during follow-up

	Rs11591147			
	Wt/Wt (n=5337)	Wt/Var (n=193)	Var/Var (n=2)	P-trend
Baseline				
Cholesterol levels				
Total cholesterol (mmol/l)	5.64 (0.03)	5.30 (0.07)	5.04 (0.59)	1.18×10^{-8}
LDL-cholesterol (mmol/l)	3.66 (0.03)	3.29 (0.06)	3.08 (0.52)	3.62×10^{-12}
Functional status				
Barthel score	19.77 (0.03)	19.70 (0.06)	19.57 (0.51)	0.21
Instrumental activities of daily living score	13.61 (0.04)	13.63 (0.08)	12.98 (0.70)	0.90
Cognitive performance				
Mini mental state examination score	28.00 (0.05)	28.05 (0.12)	28.14 (1.04)	0.67
Stroop-Colour-Word test	65.73 (0.90)	63.87 (1.97)	65.72 (16.63)	0.31
Picture Learning test immediate	9.41 (0.07)	9.26 (0.15)	10.03 (1.22)	0.32
Picture Learning test delayed	10.27 (0.09)	10.13 (0.21)	10.41 (1.74)	0.48
Letter-digit Coding test	23.44 (0.25)	23.24 (0.54)	21.39 (4.57)	0.63
Repeated cross-sectional				
Functional status				
Barthel score	19.61 (0.03)	19.55 (0.08)	19.40 (0.66)	0.37
Instrumental activities of daily living score	13.29 (0.05)	13.32 (0.10)	11.24 (0.88)	0.90
Cognitive performance				
Mini mental state examination score	28.04 (0.05)	28.12 (0.12)	28.37 (1.02)	0.40
Stroop-Colour-Word test	66.48 (0.86)	63.96 (1.89)	66.91 (17.19)	0.16
Picture Learning test immediate	9.37 (0.06)	9.29 (0.13)	9.20 (1.16)	0.51
Picture Learning test delayed	10.01 (0.08)	9.96 (0.18)	10.94 (1.63)	0.86
Letter-digit Coding test	22.85 (0.23)	23.12 (0.49)	22.45 (4.44)	0.57

Estimates and p-values at baseline were assessed with general linear model adjusted for sex, age, and country. Analyses on cognition were additional adjusted for education, APOE genotype, and were appropriate, version of test used. Repeated cross-sectional estimates and p-values were assessed with linear mixed model and additional adjusted for follow-up time. Values are means (SE).

Next, we assessed the association between rs11591147 and history of diabetes and hypertension at baseline. Carrying a variant of rs11591147 was not associated with a history of diabetes or hypertension (both p>0.4) (Table 3). Also during follow-up, there was no association between rs11591147 and the incidence of cancer, diabetes and infections (Table 3).

Table 3. Rs11591147 and history or incidence of noncardiovascular clinical events

		Rs11591147			
		Wt/Wt (n=5571)	Wt/Var (n=204)	Var/Var (n=2*)	
	N events	OR (95% CI)	OR (95% CI)	OR (95% CI)	P-trend
History of diabetes	617	1 (ref)	0.82 (0.49-1.36)	NA	0.44
History of hypertension	3575	1 (ref)	1.08 (0.80-1.45)	NA	0.63
		HR (95% CI)	HR (95% CI)	HR (95% CI)	
Serious cancer	441	1 (ref)	0.86 (0.49-1.49)	NA	0.59
Incident diabetes	291	1 (ref)	1.39 (0.81-2.39)	NA	0.23
Incident infection	313	1 (ref)	0.90 (0.48-1.68)	NA	0.73

Odds ratios (OR) and corresponding p-values were assessed with logistic regression analysis adjusted for age, sex, and country. Hazard ratios (HR) and p-values were assessed with Cox-proportional hazard models adjusted for age, sex, country, and pravastatin use.

* not possible to perform logistic or Cox regression with n=2.

To cover the whole *PCSK9* gene, we also assessed the association between other *PCSK9* tag SNPs (rs2094470, rs2479415, rs7525649, rs17111495, rs10888896, rs4927193, rs2479411, rs499718, rs10888897, rs529787, rs562556, rs615563, rs10465832, rs505151, rs662145, rs9326034) and cholesterol levels, cognitive performance, functional status, and clinical events using data of the PHASE study [18]. Three SNPs, rs2479415, rs2182833, and rs11206514, were associated with LDL-cholesterol levels (all p<0.01). However, none of these SNPs were also associated with cognitive performance, functional status, or noncardiovascular clinical events (supplementary tables 1-2). Also, when we combined the PCSK9 SNPs into haplotypes there were no associations between the haplotypes, cholesterol levels, cognitive performance, functional status, or clinical events (data not shown).

Discussion

In this study we assessed the association between the *PCSK9* SNP rs11591147, cognitive performance, functional status and noncardiovascular clinical events in the PROSPER study. No association between rs11591147 and cognitive performance, functional status or non-vascular clinical events was observed. As the SNP was significantly associated with lower cholesterol and with cardiovascular disease

protection [7;9;16], these data do not lend arguments that drugs inhibiting PCSK9 will have other (adverse) effects.

The association between the *PCSK9* SNP rs11591147 and lipid levels and vascular disease risk has been investigated previously in PROSPER [16]. In this analysis carriers of the rare variant of rs11591147 had significantly 10% lower LDL-cholesterol levels ($P<0.001$). These lower LDL-cholesterol levels are well in line with the findings of a meta-analysis including seven general population studies with a total of 1,639 carriers of the rs11591147 minor allele and 59,298 non-carriers [9]. In this meta-analysis, rs11591147 was associated with 12% lower LDL-cholesterol levels in carriers. The same article also reported a meta-analysis combining studies which investigated the risk of ischemic heart disease (IHD), including 11,339 cases and 55,359 control subjects. The rs11591147 SNP was associated with a reduction in risk of IHD of 28 percent (fixed effect OR: 0.72, 95% CI: 0.62-0.84). This effect is higher than the previously reported results in PROSPER where rs11591147 was associated with a non-significant reduced risk of vascular disease of 9%.

The LDLR plays an important role in cholesterol metabolism by mediating the uptake of apoB containing lipoprotein from the blood into liver cells [2]. PCSK9 is involved in the cholesterol metabolism by promoting the degradation of LDLR [6]. There are indications that cholesterol metabolism is associated with cognitive performance. High total serum cholesterol levels have been shown to associate with lower cerebral spinal fluid levels of β-amyloid and larger amounts of β-amyloid deposition in brain autopsy studies [11]. Since PCSK9 is involved in the cholesterol metabolism this raises the question if PCSK9 is also associated with cognitive function. A Canadian study investigated the relation between PCSK9 and Alzheimer's disease. In this study variations in rs2483205, rs483462, and rs662145 were associated with an increased Alzheimer's disease risk [10]. However, those SNPs are not known to be associated with cholesterol levels or in linkage disequilibrium with one of our SNPs associated with LDL-cholesterol. A study with Japanese Alzheimer's disease patients found no association between the PCSK9 SNPs rs11583680 and rs662145 and Alzheimer's disease [20]. In our study, rs11591147 was not associated with cognitive performance.

Since PCSK9 has high affinity for the LDLR, inhibiting PCSK9 might be a promising therapeutic target for lipid lowering therapy in addition to statin therapy. Several approaches to inhibit PCSK9 are currently under development, but it is unknown what the influences of those inhibitors will be on cognitive function or noncardiovascular events [2]. In our study rs11591147, associated with LDL-cholesterol levels, did not affect cognitive performance, functional status, or noncardiovascular

clinical events. Based on our results one may expect that drugs inhibiting PCSK9 [21-23], to an extend comparable to the rs11591147 mutation, administered to an age group 70 to 82 years with a history or increased risk of cardiovascular disease are not likely to affect cognitive performance. This suggests that cholesterol lowering by PCSK9 inhibition may be a valid and safe way to reduce cardiovascular disease risk. However, because the inhibitors in development may cause larger LDL-cholesterol reductions compared with the rs11591147 mutation, definite conclusions about the adverse effects of cholesterol lowering due to PCSK9 inhibition cannot be assessed by this study.

One of the strengths of our study is our population size. We have prospective data of over 5000 subjects on lipid parameters, cognitive function, and clinical endpoints. Also we have a follow-up of 42 months with very little lost to follow-up. Furthermore, our population is an appropriate study population to measure cognitive function, since only subjects with a MMSE above 24 points were eligible for participation, which makes it a homogenous study group suitable for investigating cognitive function.

A possible limitation of our study is the limited power to detect subtle differences. Although the PROSPER study is one of the largest studies to investigate the association between genetic variations and lipid levels, cognitive function, and clinical events, we do not have infinite power to detect small effects. But we assume that it is unrealistic that we have missed clinically relevant effects. Another possible limitation is the ceiling effect of the ADL measurements. This may have restricted our ability to find significant associations between the *PCSK9* mutation and ADL performance. However, previous analysis on apolipoprotein E genotype have proven that the PROSPER study is suitable for investigating genetic influences on cognition and ADL [24]. A last possible limitation is the selection of the PROSPER participants. Participants were recruited when they had existing vascular disease or increased risk of such disease [13]. Individuals especially susceptible to the mutation leading not only to low LDL-cholesterol but also protective for cardiovascular disease may have been under represented in our study.

In conclusion, it has previously been shown that the *PCSK9* SNP rs11591147 is associated with lower LDL-cholesterol levels and lower cardiovascular risk. In our study we failed to find associations between the variant and cognitive phenotypes. The implication of our finding is that it is unlikely that medication lowering LDL-cholesterol via inhibiting PCSK9 to an extent comparable to the effect of the

mutation in our study population, will affect cognitive performance, functional status, or noncardiovascular clinical events.

Supplementary Material

Supplementary material is available at *Journal of Lipid research* online.

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CHAPTER 10

Main findings and general discussion

Main findings and general discussion

The main aim of this thesis was to investigate pharmacogenetic effects on response to statin treatment and the genetics of lipid metabolism and cardiovascular disease (CVD). Since CVD is one of the leading causes of death worldwide, it is important to identify factors that influence the response to treatment [1;2]. Identifying genetic variants associated with response to statin treatment could possible allow for personalized treatment or the development of new drugs to reduce the burden of CVD.

Chapter 2 described the current state of the pharmacogenetic studies investigating the response to statin treatment. In previous decades mainly candidate gene studies were used to investigate the efficiency and clinical effectiveness of statin therapy. Based on these candidate gene studies, which investigated only one or a few genes per study, genes regulating pharmacokinetic and pharmacodynamic properties of statins appear to be the most promising target genes [3]. The more recent genome-wide association studies (GWAS) give the possibility to investigate the whole genome with no a priori assumptions, facilitating new discoveries. Although multiple pharmacogenetic GWAS have investigated the response to statin treatment, only two genetic loci, *APOE* and *LPA*, have been consistently found to be associated with variation in low-density lipoprotein cholesterol (LDL-C) response to statin therapy [4;5]. To assess pharmacogenetic associations within the PROSPER study we conducted the PHArmacogenetic study of Statins in the Elderly at risk (PHASE). In **chapter 3** we presented the first GWAS performed in PHASE, replicating the previously identified genetic variants associated with LDL-cholesterol levels.

Due to the large number of statistical test performed in a GWAS analysis, large sample sizes are required to provide adequate statistical power to detect small effect sizes. Therefore, to identify novel genetic variants associated with response to statin treatment, it is necessary to collaborate with other studies. In **chapter 4** the results of the first analyses performed within the Genomic Investigation of Statin Therapy (GIST) consortium were shown. More than 40,000 statin treated subjects from both randomized controlled trials and observational studies were included in the analysis. We identified and validated two novel GWAS loci associated with LDL-C response at *SORT1/CELSR2/PSRC1* and the *SLCO1B1* loci. Furthermore, we confirmed the previously described associations within the *LPA* and *APOE* gene loci. These findings advance the understanding of the pharmacogenetic architecture of statin response.

Most of the pharmacogenetic studies with regard to statin therapy have focused on the lipid lowering response, while relatively little is known about the genetic variation associated with variability in clinical event reduction by statins. As only one GWAS study had investigated this association [6], we performed a GWAS on differential event reduction by pravastatin therapy in **chapter 5**. However, we did not identify any genetic variants significantly associated at a genome-wide level with a clinically meaningful differential event reduction by pravastatin treatment in the PROSPER/PHASE study. These results indicate that in daily practice the use of genetic characteristics to personalize pravastatin treatment to improve prevention of CVD will be limited. However, as mentioned before, GWA studies require large sample sizes to provide adequate statistical power to detect small effect sizes. To increase the chance of identifying genetic variants associated with differential event reduction by statin therapy, the GIST consortium is currently performing a meta-analysis of GWAS studies investigating differential cardiovascular event reduction by statins.

Together, the genetic variants assessed within the GIST GWAS meta-analysis explained 5% of the variance in LDL-cholesterol lowering response (**chapter 4**). Whether this 5% is clinically relevant is as yet unclear and should be tested in new research studies. Within the PROSPER study we were not able to identify any genetic variant associated with a clinically meaningful differential CVD event reduction by pravastatin (**chapter 5**). Therefore we might wonder how pharmacogenetics of (prava) statin therapy can be of any clinically relevant use for individual patients in clinical care. However, newer research methods such as whole genome sequencing may reveal rare genetic variants with larger effect sizes [7]. The aim of sequencing studies is to identify rare variants, present in <1% of the population. It will therefore be difficult to apply these results in the clinical setting. Combining several genetic variants with small effects into a genetic risk score (GRS) might be another option to use pharmacogenetic research in clinical practice.

In pharmacogenetic research, non-responders to a drug are often compared to high-responders, with the aim of identifying the genetic loci responsible for this variation in response [8;9]. In **chapter 6** we tried to answer the question whether the non-responders are true non-responders or whether they are actually non-adherent. Our results suggest that many of the so called non-responders are more likely to be non-adherers, since they have more clinical characteristics related to high self-perceived health and low disease awareness. In addition, these results indicate that other strategies should be used to investigate the relation between genetic variation and responsiveness to statin treatment. In **chapter 7** we discuss whether there are

indications that statins work differently around the world. Despite the many different ethnicities worldwide, the most important risk factors for CVD are the same for, and statins seem to be similarly effective in, all studied populations around the world. However, this does not suggest that different ethnicities can be combined in pharmacogenetic studies, as allele frequencies of genetic variants may vary across ethnicities [10].

In **chapter 8** we focused on the question whether high LDL-C levels are still a risk factor for mortality in the elderly. To overcome the problems of confounding and reverse causality we used a LDL genetic risk score (GRS) based on the Mendelian randomization concept [11]. The level of this GRS decreased by increasing age and was significantly associated with mortality in the participants aged 90 years and older. In addition we showed that participants with a genetic predisposition for longevity had a lower GRS compared to participants from the general population. These results indicate that a genetic predisposition to high LDL-C contributes to mortality throughout life also in the oldest old. These results are supported by the fact that statin therapy is still beneficial in the treatment of CVD in the elderly [12].

In the first chapters of this thesis we showed that there is currently limited evidence for a role of genetics in the individual variability in statin response. In **chapter 8** we showed that a genetic predisposition to high LDL-C contributes to mortality even in the oldest old, therefore lowering LDL-C levels should remain an important goal in reducing CVD risk. New drugs to lower LDL-C levels are currently in development. One of those promising drugs are the PCSK9 inhibitors [13], however it is currently unknown what the effect of those inhibitors will be on cognition or non-cardiovascular clinical events. In **chapter 9** we investigated whether genetic variation within the *PCSK9* gene was associated with cognitive performance and non-cardiovascular clinical events in the PROSPER study. We showed that a genetic variant in the *PCSK9* gene was associated with lower LDL-C levels, but not with cognitive performance or non-cardiovascular events. This suggests that it is unlikely that lowering of cholesterol levels through the inhibition of PCSK9 will affect cognitive performance and non-cardiovascular clinical events.

Future perspectives

In the past few years, genome-wide association studies have become a popular approach for investigating associations between response to drugs and genetic variants. The ultimate aim of pharmacogenetic studies is to improve and personalize individual drug treatment [14;15]. This thesis shows that there is currently limited

evidence for a role of genetics in the individual variability in statin response. In other disease areas pharmacogenetic research has shown promising results [8]. For example, genetic variation in the cytochrome P450 enzyme CYP2C9 and vitamin K epoxide reductase genes explained up to 35% of the variability in required warfarin starting dose [16]. However, three new clinical trials assessing the role of pharmacogenetics in the dosing of vitamin K antagonists have shown disappointing results [17-19]. The results of the three trials show that the usefulness of pharmacogenetic testing for the initial dose of vitamin K antagonists is absent or marginal [20].

Although the PROSPER/PHASE study is one of the largest randomized clinical trials to investigate the pharmacogenetics of statins, the sample size might still be too small. To increase the sample size for pharmacogenetic studies, collaborating in large consortia is necessary. To this end, the GIST consortium was formed (**chapter 4**). Within this consortium the pharmacogenetic effects of statins on CVD event reduction are also investigated. Other ongoing studies investigate statin pharmacogenetics on C-reactive protein (CRP) response. Statin treatment is associated with reduction in CRP levels in a LDL-C-independent manner, reflecting the anti-inflammatory properties of statins [21]. Possible, these analyses will lead to the discovery of new genetic variants that might be beneficial in novel mechanisms/pathways for the treatment of CVD.

Recent developments such as whole genome sequencing methods and the 1000 genomes imputation will give new opportunities for the genetic research field [7;22]. Compared to methods using the HapMap haplotypes as reference panel [23], the more recent 1000 Genomes Project allows for much denser imputations [22]. However, using the 1000 Genomes imputation will probably mainly refine the previously identified associations and identify genetic variants that are in high linkage disequilibrium with the variants identified in the previous GWAS based on HapMap imputations [24]. Sequencing studies aim to identify rare variants, present in less than 1% of the population. These rare variants are not present on the chips currently used in GWAS, which contain far less genetic variants and only the more common ones. It remains the question whether the variants identified using sequencing, the rare variants with large effect sizes, will explain the missing heritability [25].

Mendelian randomization is another method increasingly used in genetic research. For example, the use of a genetic risk score has helped to unravel the question of causality with regard to HDL-C levels and CVD [26]. Based on observational studies in middle-aged populations, high LDL-C and low HDL-C levels appeared to be important risk factors for CVD [26]. LDL-C lowering treatments such as statins are therefore

effective in the reduction of the CVD risk [27]. However, thus far, increasing HDL-C levels with pharmacological therapies have not lead to clinical beneficial results [28]. Using Mendelian randomization analysis, it was shown that HDL-C was not causally associated with the risk for myocardial infarction, whereas LDL-C was [26]. Most studies investigating the association between LDL-C and CVD or mortality were performed in middle-aged populations, as was the Mendelian randomization analysis, while the few studies performed in older aged subjects showed inverse or no associations [29]. This thesis shows that a genetic predisposition to high LDL-C contributes to mortality, also in the oldest old. However, the power of this analysis was limited as the results were mainly driven by one of the three studies. To increase the evidence that high LDL-C is still a risk factor for CVD in the elderly, the LUMC will start a large meta-analysis of Mendelian randomization studies investigating a LDL genetic risk score in various age groups and assess the association with cardiovascular events.

Conclusions

LDL-cholesterol is an important risk factor for CVD, even at old age. Reducing LDL-cholesterol levels is therefore essential in the treatment of CVD. To improve the current treatment, new drugs such as the PCSK9 inhibitors, are in development. Another option to improve treatment is to personalize treatment based on an individual's genetic make-up, thereby reducing polypharmacy and the risk of adverse events. With the currently available research approaches to investigate statin pharmacogenetics, only four genetic variants have been found to be associated with the LDL-cholesterol response. As shown in this thesis, these four genetic variants explain only 5% of the observed variation in LDL-cholesterol lowering response to statins. Therefore the possibilities to personalize statin treatment based on genetic variants remain limited. New research methods will hopefully give new opportunities to improve CVD treatment and give more insight into the biological mechanisms of statin treatment.

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NEDERLANDSE SAMENVATTING

Hoofdbevindingen

Doel van dit proefschrift was het vinden van genetische varianten welke een rol spelen in de respons op statine behandeling. Daarnaast is de genetische achtergrond van het lipide metabolisme en hart- en vaatziekten (HVZ) verder uitgezocht. Aangezien HVZ een van de belangrijkste doodsoorzaken wereldwijd zijn, is het belangrijk om factoren te vinden welke de respons op behandeling beïnvloeden. Het vinden van genetische varianten geassocieerd met statine respons kan mogelijk leiden tot gepersonaliseerde behandeling of de ontwikkeling van nieuwe medicatie om de gevolgen van HVZ te verminderen.

Hoofdstuk 2 beschrijft de tot aan dat moment uitgevoerde farmacogenetische studies naar de respons op statine behandeling. In de voorgaande jaren zijn voornamelijk kandidaat gen studies uitgevoerd om de efficiëntie en klinische effectiviteit van statines te onderzoeken. Gebaseerd op de resultaten van deze studies, die maar enkele genen per studie onderzochten, lijken met name genen welke de farmacokinetische en farmacodynamische eigenschappen van statines beïnvloeden de meest belovende target genen. Met de recentere genoom wijde associatie studies (GWAS) kan het gehele genoom hypothese-vrij geanalyseerd worden, wat nieuwe ontdekkingen mogelijk maakt. Hoewel al meerdere farmacogenetische GWAS de respons op statines hebben onderzocht, zijn er maar twee genetische varianten gevonden die consistent geassocieerd zijn met de variatie in LDL-cholesterol respons. Om ook farmacogenetisch onderzoek te kunnen doen in de PROSPER studie, is de PHArmacogenetic study of Statins in the Elderly at risk (PHASE) uitgevoerd. In **hoofdstuk 3** tonen we resultaten van de eerste GWAS uitgevoerd in PHASE, hierin repliceren we de eerder gevonden associaties met LDL-cholesterol levels.

Een gevolg van het grote aantal statistische testen dat wordt uitgevoerd in een GWAS, is dat je een grote studiepopulatie nodig hebt om met genoeg statistische power kleine effecten aan te tonen. Voor het vinden van nieuwe associaties tussen genetische varianten en respons op statine behandeling is het essentieel om samen te werken met andere studies. Met dit doel is het Genomic Investigation of Statin Therapy (GIST) consortium gevormd, waarvan we in **hoofdstuk 4** de eerste resultaten presenteren. Data van meer dan 40.000 met statine behandelde personen zijn meegenomen in deze analyse. We hebben twee nieuwe GWAS loci geassocieerd met LDL-cholesterol respons gevonden en gevalideerd, de *SORT1/CELSR2/PSRC1* en *SLCO1B1* loci. Daarnaast hebben we de eerder beschreven associaties met *APOE* en

LPA bevestigd. Deze bevindingen geven meer inzicht in de farmacogenetica van statine respons.

De meeste farmacogenetische studies met betrekking tot statine therapie waren gericht op LDL-cholesterol verlaging. Daarentegen is weinig bekend over de genetische variatie geassocieerd met variabiliteit in cardiovasculaire event reductie na statine behandeling. In de PROSPER/PHASE studie konden wij geen genetische varianten vinden die geassocieerd zijn met een klinisch relevante differentiële event reductie na statine behandeling (**hoofdstuk 5**). Deze resultaten indiceren dat het nut van het gebruik van genetische informatie om statine behandeling te personaliseren in de dagelijkse praktijk klein zal zijn. Echter, zoals eerder aangegeven, is voor het uitvoeren van een GWAS een grote studiepopulatie nodig voor het genereren van voldoende statistische power om kleine effecten aan te tonen. Voor het vergroten van de kans op het vinden van genetische varianten geassocieerd met differentiële event reductie, voert het GIST consortium momenteel een meta-analyse van verschillende GWA studies uit.

De gevonden genetische varianten in de GIST GWAS meta-analyse verklaren gezamenlijk 5% van de variatie in LDL-cholesterol verlaging na statine behandeling (**hoofdstuk 4**). Of deze 5% klinisch relevant is, is nog onduidelijk en moet in nieuwe studies onderzocht worden. In de PROSPER studie waren we niet in staat om genetische varianten te vinden welke geassocieerd zijn met een klinisch relevante differentiële event afname na pravastatine behandeling (**hoofdstuk 5**). Daarom kunnen we ons afvragen of het gebruik van farmacogenetica van (prava) statine therapie enige klinische relevantie zal hebben voor de individuele patiënt. Echter, nieuwe onderzoeksmethoden zoals exome sequencing kunnen zeldzame varianten met grotere effecten aan het licht brengen. Het doel van sequencing studies is het vinden van zeldzame varianten die in minder dan 1% van de bevolking voorkomen. Waarschijnlijk zal het lastig zijn deze resultaten toe te passen in de klinische praktijk. Het combineren van verschillende genetische varianten met kleine effecten in een genetische risico score (GRS) kan een andere optie zijn voor het gebruik van farmacogenetica in de klinische praktijk.

In farmacogenetische studies worden non-responders vaak vergeleken met de high-responders, met het doel om genetische varianten te identificeren welke geassocieerd zijn met de variatie in respons. In **hoofdstuk 6** hebben we geprobeerd de vraag te beantwoorden of deze non-responders werkelijk non-responders zijn of dat zij therapie ontrouw zijn. Onze resultaten suggereren dat vele van de zogenoemde non-responders waarschijnlijk therapie ontrouw zijn, aangezien zij meer

klinische karakteristieken hebben die samen gaan met een laag ziekte bewustzijn. Verder geven deze resultaten aan dat andere strategieën gebruikt moeten worden voor het onderzoeken van de relatie tussen genetische varianten en respons op statine behandeling. In **hoofdstuk 7** bespreken we of er indicaties zijn dat statines wereldwijd anders werken. Ondanks de vele verschillende bevolkingsgroepen wereldwijd, zijn de belangrijkste risicofactoren voor HVZ gelijk en zijn statines even efficiënt in alle onderzochte bevolkingsgroepen. Dit suggereert echter niet dat verschillende bevolkingsgroepen gecombineerd kunnen worden in farmacogenetisch onderzoek, aangezien de frequentie van genetische varianten kan variëren tussen bevolkingsgroepen.

In **hoofdstuk 8** hebben we geprobeerd de vraag te beantwoorden of verhoogde LDL-cholesterol levels op hoge leeftijd nog steeds een risico factor zijn voor mortaliteit. Om problemen als confounding en omgekeerde causaliteit te voorkomen hebben we gebruik gemaakt van een LDL genetische risico score (GRS) gebaseerd op het Mendeliaanse randomisatie concept. De LDL GRS nam af met toenemende leeftijd en was significant geassocieerd met mortaliteit in de deelnemers van 90 jaar en ouder. Daarnaast hebben we aangetoond dat deelnemers met een genetische aanleg voor langlevendheid een lagere GRS hebben in vergelijking met deelnemers van de algemene populatie. Deze resultaten suggereren dat een genetische aanleg voor een hoog LDL-cholesterol ook op hoge leeftijd een risico factor is voor mortaliteit. Deze resultaten worden ondersteund door het feit dat statines ook in ouderen efficiënt zijn in de behandeling van HVZ.

In de eerste hoofdstukken van dit proefschrift hebben we laten zien dat er momenteel weinig bewijs is voor een grote rol voor de genetica in het verklaren van de individuele variatie in respons op statine behandeling. In **hoofdstuk 8** hebben we laten zien dat een genetische aanleg voor een hoog LDL-cholesterol een risico factor voor mortaliteit is, zelfs op hoge leeftijd. Daarom moet het verlagen van LDL-cholesterol levels een belangrijk doel blijven bij het verlagen van het risico op HVZ. Momenteel zijn er nieuwe medicijnen om LDL-cholesterol te verlagen in ontwikkeling. Een van de veelbelovende medicijnen zijn de PCSK9 remmers. Wel is het nog onbekend wat het effect van deze medicijnen zal zijn op de cognitie en niet-cardiovasculaire events. In **hoofdstuk 9** hebben we onderzocht of genetische varianten in het *PCSK9* gen geassocieerd zijn met de cognitieve functie en niet-cardiovasculaire events in de PROSPER studie. We hebben aangetoond dat de genetische variant in het *PCSK9* gen geassocieerd is met lagere LDL-cholesterol levels, maar niet met cognitieve functie en niet-cardiovasculaire events. Deze resultaten suggereren dat het niet waarschijnlijk is dat cholesterol verlaging door

middel van PCSK9 remmers invloed zal hebben op het cognitief functioneren of niet-cardiovasculaire events.

Toekomstperspectieven

Genoom-wijde associatie studies zijn gedurende de laatste jaren een populaire methode geworden voor het onderzoeken van associaties tussen genetische varianten en de respons op medicatie. Het ultieme doel van farmacogenetische studies is het verbeteren en personaliseren van medicijn gebruik. In dit proefschrift laten we zien dat er momenteel weinig bewijs is voor een grote rol van de genetica in het verklaren van de individuele variatie in respons op statine behandeling. Voor een aantal andere medicijnen lijken de resultaten van farmacogenetisch onderzoek veelbelovend. Genetische varianten in de cytogroom P450 enzym CYP2C9 en vitamine K epoxide reductase genen verklaren tot 35% van de benodigde warfarine startdosering. Echter, de resultaten van drie recente klinische studies gebruikmakende van genetische informatie in de bepaling van de vitamine K antagonist dosering zijn teleurstellend. Deze laten zien dat de toepasbaarheid van genetisch testen voor de initiële vitamine K antagonist dosering minimaal of afwezig is.

Hoewel de PROSPER/PHASE studie een van de grootste gerandomiseerde studies is voor het onderzoeken van statine farmacogenetica, is de populatie grootte mogelijk nog te klein. Voor het vergroten van de studiepopulatie is het samenwerken in grote consortia noodzakelijk. Om dit te bewerkstelligen is het GIST consortium gevormd (**hoofdstuk 4**). Binnen dit consortium zal ook gezocht worden naar genetische varianten die invloed hebben op de reductie van HVZ events na statine behandeling. In een andere analyse zal gezocht worden naar farmacogenetische effecten op C-reactief proteïne (CRP) respons na statine behandeling. Statine behandeling is geassocieerd met een verlaging van CRP waarden onafhankelijk van LDL-cholesterol, dit weerspiegeld de ontstekingsremmende eigenschappen van statines. Mogelijk zullen deze analyses leiden tot de ontdekking van nieuwe genetische varianten welke betrokken kunnen zijn bij nieuwe mechanismes in de behandeling van HVZ.

Recente ontwikkelingen als de sequencing van het gehele genoom en de 1000 genomen imputatie zullen nieuwe mogelijkheden bieden voor het genetisch onderzoek. Met deze nieuwe methoden kan het genoom in nog meer detail geanalyseerd worden. Maar waarschijnlijk zal het gebruik van de 1000 genomen imputatie vooral leiden tot de verfijning van de eerder gevonden associaties doordat veel van de nieuwe associaties sterk gerelateerd zullen zijn aan de eerder gevonden

associaties met de HapMap imputaties. Het doel van sequencing studies is het vinden van zeldzame genetische varianten, aanwezig in minder dan 1% van de bevolking. Deze varianten zijn niet aanwezig op de chips welke momenteel gebruikt worden voor de GWAS, welke minder varianten bevatten en alleen de gebruikelijke varianten. Het is nog de vraag of de genetische varianten welke gevonden worden met sequencing, de zeldzame varianten met grote effecten, de nog ontbrekende verklaring van erfelijkheid zullen verklaren.

In het genetisch onderzoek worden meer en meer analyses uitgevoerd die gebruik maken van Mendeliaanse randomisatie. Een genetische risico score is bijvoorbeeld gebruikt om de relatie tussen HDL-cholesterol en HVZ te onderzoeken. Gebaseerd op resultaten uit observationele studies, lijken een hoog LDL-cholesterol level en een laag HDL-cholesterol level belangrijke risico factoren te zijn voor HVZ. Bovendien zijn LDL-cholesterol verlagende behandelingen, bijvoorbeeld met statines, effectief in het verlagen van het HVZ risico. Daarentegen zijn HDL-cholesterol verhogende therapieën tot op heden nog niet effectief gebleken in de behandeling van HVZ. Door middel van Mendeliaanse randomisatie analyse is aangetoond dat HDL-cholesterol niet causaal geassocieerd is met het risico op het krijgen van een hartinfarct. LDL-cholesterol bleek hier wel causaal mee geassocieerd te zijn. De meeste studies die de associatie tussen LDL-cholesterol en HVZ of mortaliteit hebben onderzocht zijn uitgevoerd in populaties van middelbare leeftijd. De enkele studies welke uitgevoerd zijn in ouderen hebben omgekeerde of geen associaties aangetoond. In dit proefschrift hebben we door middel van een Mendeliaanse randomisatie analyse aangetoond dat ook in ouderen een genetische aanleg voor een hoog LDL-cholesterol bijdraagt aan een verhoogd mortaliteitsrisico. Echter, de power van deze analyse was beperkt, de resultaten werden met name gedreven door een van de drie studies. Om het bewijs dat LDL-cholesterol ook in de ouderen een risicofactor is te vergroten, zal er vanuit het LUMC een meta-analyse uitgevoerd worden. In deze meta-analyse zal de associatie tussen LDL-cholesterol en HVZ worden onderzocht doormiddel van een LDL-cholesterol genetische risico score in verschillende leeftijdscategorieën.

Conclusie

LDL-cholesterol is een belangrijke risico factor voor HVZ, ook op hoge leeftijd. Het verlagen van LDL-cholesterol levels is daarom belangrijk in de behandeling van HVZ. Om de huidige behandeling te verbeteren zijn nieuwe medicijnen, zoals de PCSK9 remmers, in ontwikkeling. Een andere mogelijkheid om de huidige behandeling te verbeteren, is door de behandeling te personaliseren aan de hand van iemands genetisch profiel. Door het personaliseren van de behandeling zal de polyfarmacie en

de kans op bijwerkingen verlaagd worden. Met de huidige genetische onderzoeksmethoden zijn vier genetische varianten gevonden welke geassocieerd zijn met de mate van LDL-cholesterol verlaging na statine behandeling. In dit proefschrift hebben we laten zien dat deze vier varianten slechts 5% van de geobserveerde variatie in LDL-cholesterol respons verklaren. Hierdoor zijn de mogelijkheden om statine behandeling te personaliseren op basis van iemands genetisch profiel tot op heden beperkt. Mogelijk bieden nieuwe onderzoeksmethoden nieuwe mogelijkheden tot het verbeteren van de behandeling van HVZ en geven ze meer inzicht in de biologische mechanismes van statine behandeling.

LIST OF PUBLICATIONS

1. **Postmus I**, Trompet S, Deshmukh HA, Barnes MR, Li X, Warren HR, Chasman DI, Zhou K, Arsenault BJ, Donnelly LA, Wiggins KL, Avery CL, Griffin P, Feng Q, Taylor KD, Li G, Evans DS, Smith AV, de Keyser CE, Johnson AD, de Craen AJ, Stott DJ, Buckley BM, Ford I, Westendorp RG, Slagboom PE, Sattar N, Munroe PB, Sever P, Poulter N, Stanton A, Shields DC, O'Brien E, Shaw-Hawkins S, Chen YD, Nickerson DA, Smith JD, Dubé PM, Boekholdt MS, Hovingh KG, Kastelein JJ, McKeigue PM, Betteridge J, Neil A, Durrington PN, Doney A, Carr F, Morris A, McCarthy MI, Groop L, Ahlqvist E; Welcome Trust Case Control Consortium, Bis JC, Rice K, Smith NL, Lumley T, Whitsel EA, Stürmer T, Boerwinkle E, Ngwa JS, O'Donnell CJ, Vasan RS, Wei WQ, Wilke RA, Liu CT, Sun F, Guo X, Heckbert SR, Post W, Sotoodehnia N, Arnold AM, Stafford JM, Ding J, Herrington DM, Kritchevsky SB, Eiriksdottir G, Launer LJ, Harris TB, Chu AY, Giulianini F, MacFadyen JG, Barratt BJ, Nyberg F, Stricker BH, Uitterlinden AG, Hofman A, Rivadeneira F, Emilsson V, Franco OH, Ridker PM, Gudnason V, Liu Y, Denny JC, Ballantyne CM, Rotter JI, Cupples AL, Psaty BM, Palmer CN, Tardif JC, Colhoun HM, Hitman G, Krauss RM, Jukema JW, Caulfield MJ. Pharmacogenetic meta-analysis of genome-wide association studies of LDL cholesterol response to statins. Nat Commun. 2014;5:5068.

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CM, Franceschi C, Houwing-Duistermaat JJ, Slagboom PE. Genome-wide association meta-analysis of human longevity identifies a novel locus conferring survival beyond 90 years of age. Hum Mol Genet. 2014;23(16):4420-32.

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11. Trompet S, de Craen AJ, **Postmus I**, Ford I, Sattar N, Caslake M, Stott DJ, Buckley BM, Sacks F, Devlin JJ, Slagboom PE, Westendorp RG, Jukema JW; PROSPER Study Group. Replication of LDL GWAs hits in PROSPER/PHASE as validation for future (pharmaco)genetic analyses. BMC Med Genet. 2011;12:131.

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CURRICULUM VITAE

Iris Postmus werd op 21 oktober 1986 geboren te Sneek. Na het behalen van haar VWO diploma aan het RSG Magister Alvinus te Sneek in 2005, begon zij aan de studie Biomedische Wetenschappen aan de Universiteit Utrecht. Na het behalen van haar Bachelor diploma in 2009 heeft zij haar opleiding voortgezet met de Master opleiding Biomedical Sciences aan de Universiteit Leiden. Tijdens haar eerste Master stage heeft zij bij de afdeling Trombose en Hemostase van het LUMC onderzoek gedaan naar het effect van verschillende generaties anticonceptie op de bloedstolling in muizen. Haar tweede Master stage heeft zij uitgevoerd bij de afdeling Klinische Epidemiologie van het LUMC. Hierbij heeft zij gekeken naar de relatie tussen verstoorde calcium- en fosfaat levels en de tijd tot het starten van dialyse behandeling in de PREPARE-2 studie.

Na het behalen van haar Master diploma in 2011, is zij begonnen met een promotietraject op de afdeling Ouderengeneeskunde van het LUMC. Dit promotietraject stond onder leiding van prof. J.W. Jukema (afdeling Cardiologie) en prof. R.G.J. Westendorp (afdeling Ouderengeneeskunde). De resultaten hiervan staan beschreven in dit proefschrift.

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